DESCRIPTION

A METHOD FOR IMPROVING THE THERMOSTABILITY OF α-GLUCAN PHOSPHORYLASE (GP)

TECHNICAL FIELD

The present invention relates to thermostable α-glucan phosphorylase and a gene encoding said thermostable α-glucan phosphorylase. Further, the present invention relates to a method for producing thermostable α-glucan phosphorylase.

BACKGROUND ART

α-glucan phosphorylase (hereinafter, also referred to as GP) is an enzyme utilized in, for example, synthesis of glucose-1-phosphate (hereinafter, also referred to as G-1-P), and glucan synthesis. G-1-P is utilized, for example, as a medical antibacterial agent, an anti-tumor agent (as a platinum complex), a drug for treating heart disease (as an amine salt), or a substrate for glucan synthesis. GP is widely distributed in plants, for example in tubers such as potatoes; animals, for example in rabbit muscle; and microorganisms such as yeast.

Among the above, plant-derived GP is useful because it generally has the ability to synthesize glucans having a high molecular weight.

Various GPs can be used to produce G-1-P or glucans, inter alia, potato-derived GP is used in many cases because

a relatively large amount of the enzyme is easily obtained.

In industrial production of G-1-P or a glucan using GP, it is necessary to essentially remove other enzyme activity derived from contamination of GP, particularly, phosphatase activity and amylase activity Escherichia coli and Bacillus subtilis are desirable hosts to express a GP gene when producing large amounts of GP. However, as shown in Fig.4 and Fig.5, Escherichia coli has amylase activity and phosphatase activity, and Bacillus subtilis has amylase activity. However, as shown in Figs.4 and 5, enzymes expressed by these hosts cannot be inactivated by heat treatment at 55°C, but can be almost completely inactivated by heat treatment at 60°C. Therefore, a plant-derived GP having heat resistance whereby it's activity is not lost, even after heat treatment at 60°C, has been desired.

For reference, specific numerical values of amylase activity and phosphatase activity in cell extracts from various bacteria (Escherichia coli TG-1 strain, Escherichia coli BL21 strain, and Bacillus subtilis ANA-1 strain) before and after heating are shown in the following Table 1.

(Table 1)

	Phosphatase activity (%)		Amylase activity (%)		
	TG-1	BL21	TG-1	BL21	ANA-1
Before heating	100	100	100	100	100
50°C	99.1	98.6	21.6	28.6	33.8
55°C	60.9	74.5	9.1	9.7	19.8
60°C	2.9	3.1	0.4	0	3.0
65°C	2.5	2.0	0.9	0	2.4

However, a plant-derived GP which can synthesize high molecular weight glucans, and has thermostability, particularly, GP which can maintain sufficient activity at high temperatures (e.g. 60°C to 75°C), is not known. Regarding GP derived from organisms other than plants, GP having high thermostability, GP expressed by extreme thermophilic bacteria (Thermus aquaticus, Thermococcus litoralis, Aquifex aeolicus and the like) has been reported. However, since such the above GP is derived from organisms other than plants, it is unable to synthesize high molecular weight glucans, and is thus less useful.

GPs are classified into two groups based on homology between amino acid sequences, (see Non-Patent Document 1). GP having 30% or more identity to potato-derived GP is classified as being a group A GP , and a GP having less than 30% identity to potato-derived GP and having 30% or more identity to GP of Thermus aquaticus is classified a being a group B GP.

A glucan produced using GP derived from Thermus belonging to a group B has a considerably lower molecular weight when compared with a glucan produced using potato-derived GP which is classified as a group A GP. For this reason, there is the problem that high molecular weight glucans cannot be obtained using GP derived from Thermus.

In order to solve these problems, a plant-derived GP which is advantageous for industrial utilization, and has high thermostability, is required.

Theoretical methods for making a general enzyme more thermostable, such as proline theory and amino acid

substitution based on enzyme steric structure information have been tried, but have not necessarily succeeded. For this reason, methods based upon random mutation, or methods using a combination of random mutation and theoretical methodology is currently being carried out. However, in any of these methods, every protein must be characterized by trial and error.

Regarding enzymes other than GP, it has been reported that, once the position of a particular amino acid(s) involved in improving the thermostability of an enzyme is determined, an enzyme can be made thermostable by substitution of the specified amino acid residue(s) with other amino acid residues (for example see Non-Patent Documents 3 to 5).

An example of GP having improved thermostability has been reported with regard to Escherichia coli maltodextrin phosphorylase (see Non-Patent Document 2). In this document, thermostable Escherichia coli maltodextrin phosphorylase is disclosed. Maltodextrin phosphorylase is one type of GP. In this GP having improved thermostability, asparagine at position 133 is substituted with alanine. This asparagine at position 133 is present at an active site, and is a binding site for pyridoxal 5'-phosphate which is a coenzyme essential in the enzymatic reaction. In this GP having improved thermostability, thermostability is improved by about 15°C, and the optimal reaction temperature is elevated from about 45°C to about 60°C, and the GP is denatured at about 67°C, as compared with natural GP. However, this Escherichia coli GP, similar to Thermus-derived GP, does not have the ability to synthesize high molecular weight glucans h. Further, the enzyme activity at optimal temperatures for the GP having improved thermostability described in this document, is lower

than the enzyme activity at an optimal temperature of natural GP. That is, due to mutation, the ability to synthesize a glucan thereof, is lowered. For this reason, this document teaches that substitution at position 133 is not preferable, at least from the viewpoint of glucan synthesizing ability.

Usually, an enzyme protein is unstable, and is sensitive to physical factors such as pH, temperature etc, as well as proteases, and thus may be easily degraded. Among enzymes, there are also enzymes which become more unstable, and therefore easily degraded, at high degrees of purification. For this reason, enzymes must be prepared at as low as possible temperatures, and must be prepared before every use. Degradation of an enzyme can be suppressed by freezing and storing. However, proteins are degraded upon thawing in some cases, and handling is therefore difficult when an enzyme is stored frozen and subsequently thawed. Generally, when an enzyme is degraded, the steric structure changes, and the nature of the enzyme with regard to optimal pH, pH stability, reaction rate, substrate affinity, and the like, similarly changes. Occasionally the enzyme activity is lowered, or inactivated. As such, degradation of an enzyme protein greatly influences enzyme reaction. For this reason, for industries that utilizing enzymes, it is desirable to use enzymes that have excellent stability as far as possible.

It has been known that natural potato type L GP is also easily degraded and, even when purified GP is refrigerated and stored, it gradually degrades from the point of purification. When degradation of a GP protein can be suppressed, it becomes possible to prepare a large amount of GP and store it long term, thus increasing production efficiency, which is a significant advantage in terms of

both storage and use of an enzyme. For this reason, it is also preferable to provide GP which can be stored long term, without degradation.

(Non-Patent Document 1)

Takeshi Takaha, et el., "Structure and Properties of Thermus aqaticus α-Glucan Phosphorylase Expressed in Escherichia coli", J. Appl. Glycosi., 2001, Vol.48, No.1, pp.71-78

(Non-Patent Document 2)

Richard Grieβler, et al., "Mechanism of thermal denaturation of maltodextrin phosphorylase from Escherichia coli", Biochem. J., 2000, 346, pp.255-263

(Non-Patent Document 3)

Martin Lehmann and Markus Wyss, "Engineering proteins for thermostability: the use of sequence alignments versus rational design and directed evolution", Current Opinion in Biotechnology, 2001, 12, pp.371-375

(Non-Patent Document 4)

M. Lehmann, et al., "The consensus concept for thermostability engineering of proteins", Biochemica Biophysica Acta, 2000, 1543, pp.408-415

(Non-Patent Document 5)

Junichi Miyazaki, et al., "Ancestral Residues Stabilizing 3-Isopropylmalate Dehydrogenase of an Extreme Thermophile: Experimental Evidence Supporting the Thermophilic Common Ancestor Hypothesis", J. Biochem, 2001, 129, pp.777-782

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

The present invention intends to solve the aforementioned problems, and an object of the present of invention is to provide a plant-derived α-glucan phosphorylase which has better thermostability than the conventional α-glucan phosphorylase. More particularly, an object of the present invention is to provide a plant-derived α-glucan phosphorylase having excellent storage stability in addition to thermostability.

MEANS FOR SOLVING THE PROBLEMS

As a result of diligent studies to solve the aforementioned problems, the present inventors found that a plant-derived GP having improved thermostability is obtained by substituting an amino acid residue at a particular position in the amino acid sequence of a plant-derived GP. Based on these findings, the present inventors completed the present invention.

In order to solve the aforementioned problems, the present inventors continued to intensively study and, as a result, finally found that by substituting an amino acid residue in a specific position of the amino acid sequence of a plant derived GP, made the aforementioned discovery, which resulted in completion of the present of invention based thereon.

An α-glucan phosphorylase having improved

thermostability according to the present invention is an α-glucan phosphorylase having improved thermostability which is obtained by modifying a natural α-glucan phosphorylase,

wherein the natural α-glucan phosphorylase is derived from a plant, and

the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from that of the natural α-glucan phosphorylase in at least one position selected from the group consisting of:

a position corresponding to position 4 in the motif sequence 1L: H-A-E-F-T-P-V-F-S or a position corresponding to position 4 in the motif sequence 1H: H-A-Q-Y-S-P-H-F-S;

a position corresponding to position 4 in the motif sequence 2: A-L-G-N-G-G-L-G; and

a position corresponding to position 7 in the motif sequence 3L: R-I-V-K-F-I-T-D-V or a position corresponding to position 7 in the motif sequence 3H: R-I-V-K-L-V-N-D-V; and wherein

the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is 20% or more of enzyme activity at 37°C of the α-glucan phosphorylase having improved thermostability before the heating.

In one embodiment, the natural α-glucan phosphorylase can have an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase at a position corresponding to position 4 in the motif sequence 1L; or a position corresponding to position 4 in the motif sequence 1H; or a position corresponding to position 7 in the motif sequence 3L; or a position corresponding to position 7 in the motif sequence 3H.

In one embodiment, the amino acid sequence of a natural α-glucan phosphorylase can have at least 50% identity with an amino acid sequence selected from the group consisting of position 1 to position 916 of SEQ ID NO: 2; position 1 to position 912 of SEQ ID NO: 4; position 1 to position 893 of SEQ ID NO: 6; position 1 to position 939 of SEQ ID NO: 8; position 1 to position 962 of SEQ ID NO: 10; position 1 to position 971 of SEQ ID NO: 12; position 1 to position 983 of SEQ ID NO: 14; position 1 to position 928 of SEQ ID NO: 16; position 1 to position 951 of SEQ ID NO: 18; position 1 to position 832 of SEQ ID NO: 20; position 1 to position 840 of SEQ ID NO: 22; position 1 to position 841 of SEQ ID NO: 24; position 1 to position 842 of SEQ ID NO: 26; position 1 to position 841 of SEQ ID NO: 28; and position 1 to position 838 of SEQ ID NO: 30.

In one embodiment, the amino acid sequence of a natural α-glucan phosphorylase can be encoded by a nucleic acid molecule which hybridizes under stringent conditions to a nucleic acid molecule consisting of a base sequence encoding an amino acid sequence selected from the group consisting of: position 1 to position 916 of SEQ ID NO: 2; position 1 to position 912 of SEQ ID NO: 4; position 1 to position 893 of SEQ ID NO: 6; position 1 to position 939 of SEQ ID NO: 8; position 1 to position 962 of SEQ ID NO: 10, position 1 to position 971 of SEQ ID NO: 12, position 1 to position 983 of SEQ ID NO: 14; position 1 to position 928 of SEQ ID NO: 16; position 1 to position 951 of SEQ ID NO: 18; position 1 to position 832 of SEQ ID NO: 20; position 1 to position 840 of SEQ ID NO: 22; position 1 to position 841 of SEQ ID NO: 24; position 1 to position 842 of SEQ ID NO: 26, position 1 to position 841 of SEQ ID NO: 28; and position 1 to position

838 of SEQ ID NO: 30.

In one embodiment, the natural α-glucan phosphorylase can be a type L α-glucan phosphorylase, and can have an amino acid residue which is different from that of the natural α-glucan phosphorylase in at least one position selected from the group consisting of: a position corresponding to position 4 in the motif sequence 1L; a position corresponding to position 4 in the motif sequence 2; and a position corresponding to position 7 in the motif sequence 3L.

In one embodiment, the natural α-glucan phosphorylase can be type H α-glucan phosphorylase, and can have an amino acid residue which is different from that of the natural α-glucan phosphorylase in at least one position selected from the group consisting of: a position corresponding to position 4 in the motif sequence 1H; a position corresponding to position 4 in the motif sequence 2; and a position corresponding to position 7 in the motif sequence 3H.

In one embodiment, the amino acid sequence of the natural α-glucan phosphorylase can be selected from the group consisting of: position 1 to position 916 of SEQ ID NO: 2; position 1 to position 912 of SEQ ID NO: 4; position 1 to position 893 of SEQ ID NO: 6; position 1 to position 939 of SEQ ID NO: 8; position 1 to position 962 of SEQ ID NO: 10; position 1 to position 971 of SEQ ID NO: 12; position 1 to position 983 of SEQ ID NO: 14; position 1 to position 928 of SEQ ID NO: 16; position 1 to position 951 of SEQ ID NO: 18; position 1 to position 832 of SEQ ID NO: 20; position 1 to position 840 of SEQ ID NO: 22; position 1 to position 841 of SEQ ID NO: 24; position 1 to position 842 of SEQ ID NO: 26; position 1 to position 841 of SEQ ID NO: 28; and

position 1 to position 838 of SEQ ID NO: 30.

In one embodiment, the natural α-glucan phosphorylase can be derived from potato or Arabidopsis thaliana.

In one embodiment, the α-glucan phosphorylase according to the present invention can have an amino acid residue which is different from the amino acid residue of the natural α-glucan phosphorylase in at least two positions selected from the group consisting of: a position corresponding to position 4 in the motif sequence 1L or a position corresponding to position 4 in the motif sequence 1H; a position corresponding to position 4 in the motif sequence 2, and a position corresponding to position 7 in the motif sequence 3L or a position corresponding to position 7 in the motif sequence 3H.

In one embodiment, a α-glucan phosphorylase according to the present invention can have an amino acid residue which is different from an amino acid residue of a natural α-glucan phosphorylase in a position corresponding to position 4 in the motif sequence 1L or a position corresponding to position 4 in the motif sequence 1H; a position corresponding to position 4 in the motif sequence 2; and a position corresponding to position 7 in the motif sequence 3L or a position corresponding to position 7 in the motif sequence 3H.

In one embodiment, an amino acid residue at a position corresponding to position 4 in the motif sequence 1L or a position corresponding to position 4 in the motif sequence 1H can be selected from the group consisting of I, L and V.

In one embodiment, an amino acid residue at a position corresponding to position 4 in the motif sequence 1L or a position corresponding to position 4 in the motif sequence 1H can be selected from the group consisting of I and L.

In one embodiment, an amino acid residue at a position corresponding to position 4 in the motif sequence 2 can be selected from the group consisting of A, C, D, E, G, H, I, L, M, F, S, T, V and Y.

In one embodiment, an amino acid residue at a position corresponding to position 4 in the motif sequence 2 can be selected from the group consisting of C, G, S and V.

In one embodiment, an amino acid residue at a position corresponding to position 7 in the motif sequence 3L or a position corresponding to position 7 in the motif sequence 3H can be selected from the group consisting of C, I, L, V and W.

In one embodiment, an amino acid residue at a position corresponding to position 7 in the motif sequence 3L or a position corresponding to position 7 in the motif sequence 3H can be selected from the group consisting of C, I, L and V.

In one embodiment, the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, can be 30% or more of the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, before heating.

In one embodiment, the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 65°C for 2 minutes, is 10% or more of enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, before heating.

In one embodiment, the α-glucan phosphorylase having improved thermostability can have improved storage stability as compared with natural α-glucan phosphorylase.

The method of the invention is a method for producing α-glucan phosphorylase having improved thermostability, comprising:

modifying a first nucleic acid molecule comprising a base sequence encoding a first α-glucan phosphorylase to obtain a second nucleic acid molecule comprising a modified base sequence;

making an expression vector comprising the second nucleic acid molecule;

introducing the expression vector into a cell to express α-glucan phosphorylase having improved thermostability; and

recovering the expressed α-glucan phosphorylase having improved thermostability,

wherein the first α-glucan phosphorylase is derived from a plant,

the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from an amino acid residue of the first α-glucan phosphorylase in at least one position selected from the group consisting of:

a position corresponding to position 4 in the motif sequence 1L: H-A-E-F-T-P-V-F-S or a position corresponding

to position 4 in the motif sequence 1H: H-A-Q-Y-S-P-H-F-S;

a position corresponding to position 4 in the motif sequence 2: A-L-G-N-G-G-L-G; and

a position corresponding to position 7 in the motif sequence 3L: R-I-V-K-F-I-T-D-V or a position corresponding to position 7 in the motif sequence 3H: R-I-V-K-L-V-N-D-V; and wherein

the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is 20% or more of the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, before heating.

In one embodiment, an amino acid residue of the α-glucan phosphorylase having improved thermostability at a position corresponding to position 4 in the motif sequence 1L or a position corresponding to position 4 in the motif sequence 1H; or a position corresponding to position 7 in the motif sequence 3L or a position corresponding to position 7 in the motif sequence 3H, can be different from the amino acid residue of the first α-glucan phosphorylase.

In one embodiment, the first α-glucan phosphorylase can be a type L α-glucan phosphorylase, and can have an amino acid residue which is different from that of the natural α-glucan phosphorylase in at least one position selected from the group consisting of: a position corresponding to position 4 in the motif sequence 1L; a position corresponding to position 4 in the motif sequence 2; and a position corresponding to position 7 in the motif sequence 3L.

In one embodiment, the first α-glucan phosphorylase can be a type H α-glucan phosphorylase, and can have an amino

acid residue which is different from that of the natural α-glucan phosphorylase in at least one position selected from the group consisting of: a position corresponding to position 4 in the motif sequence 1H; a position corresponding to position 4 in the motif sequence 2; and a position corresponding to position 7 in the motif sequence 3H.

In one embodiment, the first α-glucan phosphorylase can be derived from potato or Arabidopsis thaliana.

A nucleic acid molecule of the present invention comprises a base sequence encoding the α-glucan phosphorylase having improved thermostability.

A vector of the present invention comprises the nucleic acid molecule.

A cell of the present invention comprises the nucleic acid molecule.

A method of synthesizing a glucan of the invention comprises reacting a reaction solution containing the α-glucan phosphorylase having improved thermostability, a sucrose phosphorylase, sucrose, a primer, and inorganic phosphoric acid or glucose-1-phosphate to produce a glucan.

In one embodiment, the reaction can be performed at a temperature of 60°C to 75°C.

Another method of synthesizing a glucan of the invention comprises reacting a reaction solution containing the α-glucan phosphorylase having improved thermostability, a primer, and glucose-1-phosphate to produce a glucan.

In one embodiment, the reaction can be performed at a temperature of 60°C to 75°C.

A method of synthesizing glucose-1-phosphate of the invention comprises reacting a reaction solution containing α-glucan phosphorylase having improved thermostability according to claim 1, a glucan and inorganic phosphoric acid to produce glucose-1-phosphate.

In one embodiment, the reaction can be performed at a temperature of 60°C to 75°C.

The α-glucan phosphorylase having improved thermostability according to the present invention is an α-glucan phosphorylase having improved thermostability which is obtained by modifying a plant-derived natural α-glucan phosphorylase,

wherein the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase at;

a position corresponding to position 4 in the motif sequence 1L: H-A-E-F-T-P-V-F-S or a position corresponding to position 4 in the motif sequence 1H: H-A-Q-Y-S-P-H-F-S;

a position corresponding to position 4 in the motif sequence 2L: A-L-G-N-G-G-L-G; and

a position corresponding to position 7 in the motif sequence 3L: R-I-V-K-F-I-T-D-V or a position corresponding to position 7 in the motif sequence 3H: R-I-V-K-L-V-N-D-V;

wherein the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is

20% or more of enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, before heating, and

the α-glucan phosphorylase having improved thermostability has the ability to synthesize an amylose having a weight average molecular weight of 600 kDa or more.

Another α-glucan phosphorylase having improved thermostability according to the present invention is an α-glucan phosphorylase having improved thermostability which is obtained by modifying a natural α-glucan phosphorylase,

wherein the natural α-glucan phosphorylase is derived from a plant,

the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from that of the natural α-glucan phosphorylase in at least one position selected from the group consisting of: a position corresponding to phenylalanine at position 39 (F39); a position corresponding to asparagine at position 135 (N135); and a position corresponding to threonine at position 706 (T706) of an amino acid sequence of SEQ ID NO: 2; and

wherein the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is 20% or more of enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, before heating.

In one embodiment, the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase at a position corresponding to phenylalanine at position 39 (M39); or a position corresponding to threonine at position 706 (T706) in an amino acid sequence of SEQ ID

NO: 2.

In one embodiment, an amino acid sequence of the natural α-glucan phosphorylase has at least 50% identity to an amino acid sequence selected from the group consisting of: position 1 to position 916 of SEQ ID NO: 2; position 1 to position 912 of SEQ ID NO:4; position 1 to position 893 of SEQ ID NO: 6; position 1 to position 939 of SEQ ID NO:8; position 1 to position 962 of SEQ ID NO: 10; position 1 to position 971 of SEQ ID NO:12; position 1 to position 983 of SEQ ID NO: 14; position 1 to position 928 of SEQ ID NO: 16; position 1 to position 951 of SEQ ID NO: 18; position 1 to position 832 of SEQ ID NO:20; position 1 to position 840 of SEQ ID NO: 22; position 1 to position 841 of SEQ ID NO:24; position 1 to position 842 of SEQ ID NO: 26; position 1 to position 841 of SEQ ID NO:28; and position 1 to position 838 of SEQ ID NO: 30.

In one embodiment, the amino acid sequence of the natural α-glucan phosphorylase is encoded by a nucleic acid molecule which hybridizes under stringent condition to a nucleic acid molecule consisting of a base sequence encoding an amino acids sequence selected from the group consisting of: position 1 to position 916 of SEQ ID NO: 2; position 1 to position 912 of SEQ ID NO:4; position 1 to position 893 of SEQ ID NO: 6; position 1 to position 939 of SEQ ID NO:8; position 1 to position 962 of SEQ ID NO: 10; position 1 to position 971 of SEQ ID NO:12; position 1 to position 983 of SEQ ID NO: 14; position 1 to position 928 of SEQ ID NO: 16; position 1 to position 951 of SEQ ID NO: 18; position 1 to position 832 of SEQ ID NO:20; position 1 to position 840 of SEQ ID NO: 22 position 1 to position 841 of SEQ ID NO:24; position 1 to position 842 of SEQ ID NO: 26; position

1 to position 841 of SEQ ID NO:28; and position 1 to position 838 of SEQ ID NO: 30.

In one embodiment, the base sequence is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO. 25, SEQ ID NO: 27 and SEQ ID NO: 29.

In one embodiment, the natural α-glucan phosphorylase is a type L α-glucan phosphorylase.

In one embodiment, the natural α-glucan phosphorylase is a type H α-glucan phosphorylase.

In one embodiment, an amino acid sequence of the natural α-glucan phosphorylase is selected from the group consisting of: position 1 to position 916 of SEQ ID NO: 2; position 1 to position 912 of SEQ ID NO:4; position 1 to position 893 of SEQ ID NO: 6; position 1 to position 939 of SEQ ID NO:8; position 1 to position 962 of SEQ ID NO: 10; position 1 to position 971 of SEQ ID NO:12; position 1 to position 983 of SEQ ID NO: 14; position 1 to position 928 of SEQ ID NO: 16; position 1 to position 951 SEQ ID NO: 18; position 1 to position 832 of SEQ ID NO:20; position 1 to position 840 of SEQ ID NO: 22; position 1 to position 841 of SEQ ID NO:24; position 1 to position 842 of SEQ ID NO: 26; position 1 to position 841 of SEQ ID NO:28; and position 1 to position 838 of SEQ ID NO: 30.

In one embodiment, the natural α-glucan phosphorylase is derived from potato or Arabidopsis thaliana.

In one embodiment, the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least two positions selected from the group consisting of: a position corresponding to phenylalanine at position 39 (F39); a position corresponding to asparagine at position 135 (N135); and a position corresponding to threonine at position 706 (T706) in an amino acid sequence of SEQ ID NO: 2.

In one embodiment, the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase at a position corresponding to phenylalanine at position 39 (F39); a position corresponding to asparagine at position 135 (N135); and a position corresponding to threonine at position 706 (T706) in an amino acid sequence of SEQ ID NO: 2.

In one embodiment, an amino acid residue at a position corresponding to the F39 is selected from the group consisting of isoleucine, valine and leucine.

In one embodiment, an amino acid residue at a position corresponding to F39 is isoleucine or leucine.

In one embodiment, an amino acid residue at a position corresponding to the N135 is selected from the group consisting of alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, valine and tyrosine.

In one embodiment, an amino acid residue at a position corresponding to N135 is cysteine, glycine, serine or valine.

In one embodiment, an amino acid residue at a position corresponding to T706 is selected from the group consisting of cysteine, isoleucine, leucine, valine and tryptophan.

In one embodiment, an amino acid residue at a position corresponding to T706 is cysteine, isoleucine, leucine or valine.

In one embodiment, the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is 30% or more of enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, before heating.

In one embodiment, the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 65°C for 2 minutes, is 10% or more of enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, before heating.

A method for producing an α-glucan phosphorylase having improved thermostability according to the present invention comprises modifying a first nucleic acid molecule comprising a base sequence encoding first α-glucan phosphorylase to obtain a second nucleic acid molecule comprising a modified base sequence; preparing an expression vector comprising the second nucleic acid molecule; introducing the expression vector into a cell to express an α-glucan phosphorylase having improved thermostability, and recovering the expressed

α-glucan phosphorylase having improved thermostability, wherein the first α-glucan phosphorylase is derived from a plant, the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from an amino acid residue of the first α-glucan phosphorylase in at least one position selected from the group consisting of a position corresponding to phenylalanine at position 39 (F39), a position corresponding to asparagine at position 135 (N135) and a position corresponding to threonine at position 706 (T706) in the amino acid sequence set forth in SEQ ID NO: 2, and wherein the enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is 20% or more of enzyme activity at 37°C of the α-glucan phosphorylase having improved thermostability at 37°C, before heating.

In one embodiment, an amino acid residue of the α-glucan phosphorylase having improved thermostability at a position corresponding to phenylalanine at position 39 (F39); or a position corresponding to threonine at position 706 (T706); in the amino acid sequence offset forth in SEQ ID NO: 2, is different from an amino acid residue of the first α-glucan phosphorylase.

In one embodiment, the first α-glucan phosphorylase is a type L α-glucan phosphorylase.

In one embodiment, the first α-glucan phosphorylase is a type H α-glucan phosphorylase.

In one embodiment, the first α-glucan phosphorylase is derived from potato or Arabidopsis thaliana.

A nucleic acid molecule of the present invention comprises a base sequence encoding the α-glucan phosphorylase having improved thermostability.

A vector of the present invention comprises the nucleic acid molecule.

A cell of the present invention comprises the nucleic acid molecule.

A method of synthesizing a glucan of the present invention comprises reacting a reaction solution containing the α-glucan phosphorylase having improved thermostability, a sucrose phosphorylase, sucrose, a primer, inorganic phosphoric acid or glucose-1-phosphate to produce a glucan.

In one embodiment, the reaction is performed at a temperature of 60°C to 75°C.

A method of synthesizing a glucan of the present invention comprises reacting a reaction solution containing the α-glucan phosphorylase having improved thermostability, a primer, and glucose-1-phosphate.

In one embodiment, the reaction is performed at a temperature of 60°C to 75°C.

A method of synthesizing glucose-1-phosphate of the present invention comprises reacting a reaction solution containing the α-glucan phosphorylase having improved thermostability, a glucan and inorganic phosphoric acid to produce glucose-1-phosphate.

In one embodiment, the reaction is performed at a temperature of 60°C to 75°C.

An α-glucan phosphorylase having improved thermostability according to the present invention is an α-glucan phosphorylase having improved thermostability obtained by modifying a plant-derived natural α-glucan phosphorylase, wherein the α-glucan phosphorylase having improved thermostability has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least one position selected from the group consisting of: a position corresponding to phenylalanine at position 39 (F39); a position corresponding to asparagine at position 135 (N135); and a position corresponding to threonine at position 706 (T706) in the amino acid sequence offset forth in SEQ ID NO: 2, wherein the enzyme activity of α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is 20% or more of enzyme activity of α-glucan phosphorylase having improved thermostability at 37°C, before heating, and the α-glucan phosphorylase having improved thermostability has the ability to synthesize amylase having an weight average molecular weight of 600 kDa or more.

EFFECT OF THE INVENTION

According to the present invention, a plant-derived GP enzyme having excellent thermostability at high temperatures, (e.g. 60°C or higher) was obtained.

According to the α-glucan phosphorylase having improved

thermostability of the present invention, a glucan synthesizing reaction is possible at high temperature conditions (e.g. 60°C or higher), under which natural GP enzymes cannot react.

The claimed invention attains the advantage that, when a gene encoding the α-glucan phosphorylase having improved thermostability of the present invention (e.g. a gene encoding GP having improved thermostability, obtained by improving thermostability of potato-derived GP) is highly expressed in mesophilic bacterium host, such as Escherichia coli, contaminating enzymes derived from the host bacterium can be simply removed by heating the bacterial cell extract containing an enzyme having improved thermostability at 60°C according to the present invention. In particular, amylase activity and phosphatase activity, which pose great problems during industrial utilization of GP enzymes, can be considerably reduced by heat treatment. Therefore, the method of the present invention is advantageous in terms of enzyme purification.

The method of the present invention is effective not only in potato-derived GP and Arabidopsis thaliana-derived GP, but can also be suitably applied to improving the thermostability of other group A GP, exhibiting high homology to an amino acid sequence of potato-derived GP or Arabidopsis thaliana-derived GP.

Therefore, other organism-derived GP having improved thermostability which has an amino acid residue which is different from that of a natural α-glucan phosphorylase in at least one position selected from the group consisting of:

a position corresponding to position 4 in the motif sequence 1L: H-A-E-F-T-P-V-F-S or a position corresponding to position 4 in the motif sequence 1H: H-A-Q-Y-S-P-H-F-S;

a position corresponding to position 4 in the motif sequence 2: A-L-G-N-G-G-L-G; and

a position corresponding to position 7 in the motif sequence 3L: R-I-V-K-F-I-T-D-V or a position corresponding to position 7 in the motif sequence 3H: R-I-V-K-L-V-N-D-V can be obtained.

Other organism-derived GP having improved thermostability can be obtained, which have an amino acid residue which is different from an amino acid residue of a natural α-glucan phosphorylase in at least one position selected from the group consisting of: a position corresponding to phenylalanine at position 39 (F39); a position corresponding to asparagine at position 135 (N135); and a position corresponding to threonine at position 706 (T706) in an amino acid sequence of SEQ ID NO: 2.

According to the present invention, GP having improved thermostability which has improved storage stability is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A: Fig. 1A is a view showing the amino acid sequences of α-glucan phosphorylases derived from various plants, which were aligned using multiple alignment of GENETYX-Win Ver.4.0.

Fig. 1B: Fig. 1B is a continuation view from Fig. 1A. Positions of motif sequences 1 and 2 are shown.

Fig. 1C: Fig. 1C is a continuation view from Fig. 1B.

Fig. 1D: Fig. 1D is a continuation view from Fig. 1C.

Fig. 1E: Fig. 1E is a continuation view from Fig. 1D.

Fig. 1F: Fig. 1F is a continuation view from Fig. 1E.

Fig. 1G: Fig. 1G is a continuation view from Fig. 1F. The position of motif sequence 3 is shown.

Fig. 1H: Fig. 1H is a continuation view from Fig. 1G.

Fig. 1I: Fig. 1I is a continuation view from Fig. 1H.

Fig. 2: Fig. 2 is a schematic view of an insertion site of an α-glucan phosphorylase gene in a plasmid.

Fig. 3: Fig. 3 is a graph showing remaining enzymatic activity (%) when various α-glucan phosphorylases having improved thermostability are incubated at 60°C for 30 minutes or at 65°C for 2 minutes.

Fig. 4: Fig. 4 is a graph showing remaining enzymatic activity (%) of phosphatase after various bacteria (Escherichia coli TG-1 and Escherichia coli BL21) are heated at 50°C, 55°C, 60°C or 65°C for 30 minutes.

Fig. 5: Fig. 5 is a graph showing remaining enzymatic activity (%) of amylase after various bacteria (Escherichia coli TG-1, Escherichia coli BL21 and Bacillus subtilis ANA-1) are heated at 50°C, 55°C, 60°C or 65°C for 30 minutes.

Fig. 6: Fig. 6 is a graph showing a change in specific enzymatic activity over time of a GP enzyme having improved thermostability (triple mutant (F39L+N135S+T706I)), and a natural potato type L GP enzyme.

Fig. 7: Fig. 7 is a graph showing the amount of amylose synthesized when a GP enzyme having improved thermostability (triple mutant (F39L+N135S+T706I)) and a natural potato type L GP enzyme are retained at 37°C, 50°C, 55°C or 60°C for 18 hours.

Fig. 8: Fig. 8 is a graph showing remaining activity after natural potato type L GP and GPs, substituted with various amino acids at F39, are incubated at 60°C for 10 minutes or at 65°C for 2 minutes.

Fig. 9: Fig. 9 is a graph showing remaining activity after natural potato type L GP and GPs, substituted with various amino acids at N135, are incubated at 60°C for 10 minutes or at 65°C for 2 minutes.

Fig. 10: Fig. 10 is a graph showing remaining activity after natural potato type L GP and GPs, substituted with various amino acids at T706, are incubated at 60°C for 10 minutes or at 65°C for 2 minutes.

Fig. 11: Fig. 11 is a graph showing remaining activity after natural potato type H GP and triple mutant (Y36L+N133S+T628I) potato type H GP are incubated at 58°C for 10 minutes, 60°C for 10 minutes or at 65°C for 2 minutes.

Fig. 12: Fig. 12 is a graph showing remaining enzymatic activity after natural Arabidopsis thaliana type H GP and

triple mutant (Y40L+N136S+N631I) Arabidopsis thaliana type H GP are incubated at 58°C for 10 minutes, at 60°C for 10 minutes or at 65°C for 2 minutes.

Fig. 13: Fig. 13 is a polyacrylamide gel electrophoresis photograph, showing the molecular weights of natural potato type L GP and seven kinds of GPs having improved thermostability immediately after purification and after storage at 4°C for 5 months. Lane 1 indicates natural potato type L (Wild type) GP, lane 2 indicates F39L GP, lane 3 indicates N135S GP, lane 4 indicates T706I GP, lane 5 indicates F39L+N135S GP, lane 6 indicates F39L+T706I GP, lane 7 indicates N135S+T706I GP, and lane 8 indicates F39L+N135S+T706I GP.

BESTS MODE FOR CARRYING OUT THE INVENTION

The present invention will be explained below. It should be understood that throughout the present specification, terms used in the present specification are used so as to have the meanings normally used in the art, unless otherwise specifically indicated.

(1. α-glucan phosphorylase)

In the present specification, "α-glucan phosphorylase" and "GP" are used exchangeably unless otherwise specifically indicated, and mean an enzyme having α-glucan phosphorylase activity. α-glucan phosphorylase is classified into EC2.4.1.1. α-glucan phosphorylase activity refers to the activity of catalyzing a reaction making glucose-1-phosphate and a partial degradation product of α-1,4-glucan from inorganic phosphoric acid and α-1,4-glucan, or a reverse

reaction thereof. α-glucan phosphorylase is called phosphorylase, starch phosphorylase, glycogen phosphorylase, maltodextrin phosphorylase, and the like, in some cases. α-glucan phosphorylase can also catalyze a α-1,4-glucan synthesis reaction which is the reverse reaction of phosphorolysis. In which direction any particular reaction progresses depend on the amount of a substrate. In vivo, since the amount of inorganic phosphoric acid is large, the reaction of glucan phosphorylase proceeds towards the direction of phosphorolysis. When the amount of inorganic phosphoric acid is small, the reaction proceeds towards the synthesis of α-1,4-glucan.

It seems that all known α-glucan phosphorylases need pyridoxal 5'-phosphate for activation, and share a similar catalytic mechanism. Although enzymes derived from different origins are different with respect to preference of substrate and form of regulation, all α-glucan phosphorylases belong to a large group including many α-glucan phosphorylases. This large group includes glycogen phosphorylase derived from bacteria, yeast and animals, starch phosphorylase derived from plants, and maltodextrin phosphorylase derived from bacteria.

It has been reported that a minimum primer molecule for a glucan synthesis reaction of α-glucan phosphorylase is maltotetraose. It has been also reported that a minimum substrate effective for a glucan degradation reaction is maltopentaose. Generally, it has been thought that these are characteristics common to α-glucan phosphorylases. However, in recent years, it has been reported that α-glucan phosphorylase derived from Thermus thermophilus and α-glucan phosphorylase derived from Thermococcus litoralis have

different substrate specificity from that of other α-glucan phosphorylases. Regarding these α-glucan phosphorylases, a minimum primer for glucan synthesis is maltotriose, and a minimum substrate for glucan degradation is maltotetraose.

It is thought that α-glucan phosphorylase is ubiquitously present in various plants, animals and bacteria which can store starch or glycogen.

Examples of a plant producing α-glucan phosphorylase include root and tuber crops such as potatoes (also referred to as Irish potato), sweet potatoes, yam, taro, and cassava; vegetables such as cabbage, and spinach; cereals such as corn, rice, wheat, barley, rye, and foxtail millet; beans such as Fava beans, peas, soybeans, adzuki beans, and mottled kidney beans; experimental plants such as Arabidopsis thaliana; Citrus hybrid cultivar, algae, and the like.

An organism producing α-glucan phosphorylase is not limited to the above examples.

It is preferable that a first α-glucan phosphorylase used in the method of the present invention is a natural α-glucan phosphorylase, and is derived from a plant. Generally, natural α-glucan phosphorylase derived from a plant has the ability to synthesize amylose having a high molecular weight. However, the thermostability of these α-glucan phosphorylases is low. For this reason, they cannot catalyse reactions at high temperatures (e.g. about 60°C or higher). For this reason, when a reaction is performed at about 30°C to about 40°C, which is the optimal reaction temperature of GP derived from plants (e.g. potato), the problem of contamination with various microbes or aging of

the glucan arises, and glucan or G-1-P can not be effectively produced.

Plant α-glucan phosphorylases are classified into types L and type H, depending on their affinity for glycogen. Type L α-glucan phosphorylase refers to α-glucan phosphorylases having a low affinity for glycogen. Generally, type L α-glucan phosphorylases prefer maltodextrin, amylase and amylopectin over glycogen as a substrate (Hiroyuki Mori, et al., "A Chimeric α-Glucan phosphorylase of Plant Type L and H Isozymes", The Journal of Biological Chemistry, 1993, vol.268, No.8, pp.5574-5581). Type H α-glucan phosphorylase refers to α-glucan phosphorylases having high affinity for glycogen. Generally, type H α-glucan phosphorylases have extremely high affinity for various glucans, including glycogen.

For example, according to Toshio Fukui, et al., Biochemistry of Vitamin B_6, 1987, pp.267-276, the K_m (Michaelis constant) of potato leaf-derived type L α-glucan phosphorylase for glycogen is 1.4×10^{-3}(M), while the K_m of potato leaf-derived type H α-glucan phosphorylase for production of glycogen is 4×1^{-6}(M). In addition, the K_m of a main component of potato tuber-derived α-glucan phosphorylase for production of glycogen is 2.4×10^{-3}(M), and this is classified as type L. The K_m of a minor component α-glucan phosphorylase for production of glycogen is 1×10^{-6}(M), and this is classified as type H.

As known in the art, the Michaelis constant is one of the kinetic parameters determined from the dependency of an initial rate in an enzymatic reaction on substrate concentration. The Michaelis constant is the substrate

concentration at a time when the initial rate becomes 1/2 the maximum rate, V_{max}. The Michaelis constant has the units of concentration. The Michaelis constant is peculiar to a enzyme under a specific set of measurement conditions. This constant is a measure indicating the affinity of an enzyme for a substrate. As a Michaelis constant becomes smaller, affinity for a substrate becomes greater.

Type L α-glucan phosphorylase and type H α-glucan phosphorylase have, for example, the following difference in properties.

(Table 2)

	Type L GP	Type H GP
Cross reactivity of antibody to a main component of potato tuber-derived GP	Presence	Absence
Cross reactivity of antibody to a minor component of potato tuber-derived GP	Absence	Presence
Sensitivity to proteolysis	High	Low
Location	Plastid (amyloplast or chloroplast)	Cytosol

In a particular embodiment, it is further preferable that an α-glucan phosphorylase used in the method according to the present invention is a type L α-glucan phosphorylase. Potato Type L α-glucan phosphorylase is longer than potato type H glucan phosphorylase, and comprises an amino acid sequence of 78 residues not seen in type H, inserted into a central region of the polypeptide chain. For this reason, for example, the molecular weight of a subunit of potato

leaf-derived type L α-glucan phosphorylase is about 104,000 Da, and the molecular weight of a subunit of potato leaf-derived type H α-glucan phosphorylase is about 94,000 Da. The molecular weight of a subunit of a main component of potato tuber-derived α-glucan phosphorylase is about 104,000 Da, and the molecular weight of a subunit of a minor component of potato tuber-derived α-glucan phosphorylase is about 94,000 Da. Whether a particular α-glucan phosphorylase is type L or type H can be determined by the presence of a region homologous with this amino acid sequence of 78 residues, without actually measuring affinity.

Generally, type L and type H are determined by comprehensively reviewing a number of properties such as enzyme activity, molecular weight, substrate specificity, location of enzyme, homology of a primary sequence, and the presence of an inserted sequence. Therefore, generally, a boundary between type L and type H is not clear in some cases, but for convenience, in the present invention, whether α-glucan phosphorylase is a type L or type H can be determined by the presence of a transit peptide in α-glucan phosphorylase. The characteristics of a transit peptide sequence are known in the art. Sequences that encode a transit peptide are type L, and sequences that do not encode a transit peptide are type H.

Examples of plants producing type L α-glucan phosphorylase include potatoes (also refers to as Irish potatoes), sweet potatoes, Fava beans, Arabidopsis thaliana, spinach, corn and rice.

In another embodiment, a first (natural) α-glucan phosphorylase used in the method of the present invention

is preferably a type H α-glucan phosphorylase. Examples of plants producing type H α-glucan phosphorylase include potatoes, wheat, Citrus hybrid cultivar, rice, Fava beans, Arabidopsis thaliana, and sweet potatoes.

The cDNA sequence of a natural type L α-glucan phosphorylase, derived from potato, is set forth in SEQ ID NO: 1, and the amino acid sequence encoded thereby is set forth in position 1 to position 916 of SEQ ID NO: 2.

The cDNA sequence of a natural type L α-glucan phosphorylase, derived from sweet potato, is set forth in SEQ ID NO: 3, and the amino acid sequence encoded thereby is set forth in position 1 to position 912 of SEQ ID NO: 4.

The cDNA sequence of another natural type L α-glucan phosphorylase, derived from potato, is set forth in SEQ ID NO: 5, and the amino acid sequence encoded thereby is set forth in position 1 to position 893 of SEQ ID NO: 6.

A cDNA sequence of a natural type L α-glucan phosphorylase, derived from Fava bean, is set forth in SEQ ID NO: 7, and the amino acid sequence encoded thereby is set forth in position 1 to position 939 of SEQ ID NO: 8.

The cDNA sequence of a natural type L α-glucan phosphorylase, derived from of Arabidopsis thaliana, is set forth in SEQ ID NO: 9, and the amino acid sequence encoded thereby is set forth in position 1 to position 962 of SEQ ID NO: 10.

The cDNA sequence of a natural type L α-glucan

phosphorylase, derived from spinach, is set forth in SEQ ID NO: 11, and the amino acid sequence encoded thereby is set forth in position 1 to position 971 of SEQ ID NO: 12.

The cDNA sequence of a natural type L α-glucan phosphorylase, derived from corn is set forth in SEQ ID NO: 13, and the amino acid sequence encoded thereby is set forth in position 1 to position 983 of SEQ ID NO: 14.

The cDNA sequence of a natural type L α-glucan phosphorylase, derived from rice, is set forth in SEQ ID NO: 15, and the amino acid sequence encoded thereby is set forth in position 1 to position 928 of SEQ ID NO: 16.

The cDNA sequence of another natural type L α-glucan phosphorylase, derived from rice, is set forth in SEQ ID NO: 17, and the amino acid sequence encoded thereby is set forth in position 1 to position 951 of SEQ ID NO: 18.

The cDNA sequence of a natural type H α-glucan phosphorylase, derived from wheat, is set forth in SEQ ID NO: 19, and the amino acid sequence encoded thereby is set forth in position 1 to position 832 of SEQ ID NO: 20.

The cDNA sequence of a natural type H α-glucan phosphorylase, derived from a Citrus hybrid cultivar, is set forth in SEQ ID NO: 21, and the amino acid sequence encoded thereby is set forth in position 1 to position 840 of SEQ ID NO: 22.

The cDNA sequence of a natural type H α-glucan phosphorylase, derived from rice, is set forth in SEQ ID NO: 23, and the amino acid sequence encoded thereby is set

forth in position 1 to position 841 of SEQ ID NO: 24.

The cDNA sequence of a natural type H α-glucan phosphorylase, derived from Fava bean, is set forth in SEQ ID NO: 25, and the amino acid sequence encoded thereby is set forth in position 1 to position 842 of SEQ ID NO: 26.

The cDNA sequence of a natural type H α-glucan phosphorylase, derived from Arabidopsis thaliana, is set forth in SEQ ID NO: 27, and the amino acid sequence encoded thereby is set forth in position 1 to position 841 of SEQ ID NO: 28.

The cDNA sequence of a natural type H α-glucan phosphorylase, derived from potato is set forth in SEQ ID NO: 29, and the amino acid sequence encoded thereby is set forth in position 1 to position 838 of SEQ ID NO: 30.

The partial sequence of a cDNA of a natural type H α-glucan phosphorylase, derived from sweet potato is set forth in SEQ ID NO: 31, and the amino acid sequence encoded thereby is set forth in SEQ ID NO: 32. A complete sequence of a natural type H α-glucan phosphorylase, derived from sweet potato, can be obtained by conventional methods using this partial sequence.

A first (natural) α-glucan phosphorylase used in the method according to the present invention is preferably derived from a plant, and is preferably derived from potato, sweet potato, Fava bean, Arabidopsis thaliana, spinach, corn, rice, wheat or a Citrus hybrid cultivar, is more preferably derived from potato, sweet potato, Fava bean, Arabidopsis thaliana, spinach, corn or rice, and is most preferably

derived from potato. It is preferable that the first (natural) α-glucan phosphorylase used in the method according to the present invention is a type L α-glucan phosphorylase. The first (natural) α-glucan phosphorylase used in the method of the present invention is preferably an α-glucan phosphorylase of type L, L2 or H derived from potato, type L or H derived from sweet potato, type L or H derived from Fava bean, type L or H derived from Arabidopsis thaliana, type L derived from spinach, type L derived from corn, type L or H derived from rice, type H derived from wheat, or type H derived from a Citrus hybrid cultivar, is more preferably α-glucan phosphorylase of type L or L2 derived from potato, type L derived from sweet potato, type L derived from Fava bean, type L derived from Arabidopsis thaliana, type L derived from spinach, type L derived from corn, or type L derived from rice, and is most preferably α-glucan phosphorylase of type L derived from potato.

In the present specification, an enzyme "derived from" an organism, means not only that the enzyme is directly isolated from the organism, but also refers to an enzyme obtained by utilizing the organism in any form. For example, when a gene encoding an enzyme obtained from an organism is introduced into Escherichia coli, and the expressed enzyme is subsequently isolated from Escherichia coli, the enzyme is referred to as being "derived from" the organism.

A gene encoding potato-derived type L GP can be prepared, for example, by the following procedure.

Firstly, as described by Takaha et al. (Journal of Biological Chemistry, Vol.268, pp. 1391-1396, 1993), an mRNA is prepared from a potato tuber using well-known methods,

and a cDNA library is prepared using a commercially available kit, and the like.

Then, based on the known GP gene sequence (database GenBank accession number D00520), PCR primers are prepared, and PCR is performed using the aforementioned cDNA library as a template. For example, when:

PCR primer 1: 5'AAATCGATAGGAGGAAAACAT ATG ACC TTG AGT GAG AAA AT 3' (SEQ ID NO: 38)

and

PCR primer 2: 5'GAAGGTACCTTTTCATTCACTTCCCCCTC3' (SEQ ID NO: 39)

are used as PCR primers, a gene can be amplified under the following conditions.

30 cycles of a PCR reaction is performed, one cycle being 30 seconds at 94°C, 1 minute at 50°C, and 3 minutes at 72 °C.

The underlined portion of the PCR primer 1 corresponds to a structural gene sequence at the N-terminal region of a type L GP mature protein, and the underlined portion of the PCR primer 2 corresponds to the base sequence immediately after the termination codon of a type L GP structural gene.

Alternatively, a GP gene can be also prepared directly by chemical synthesis, based on the known GP gene sequence information, without preparation of a cDNA library. A method of synthesizing a gene is described, for example, in Te'o,

et al. (FEMS Microbiological Letters, vol.190, pp.13-19, 2000).

The resulting GP gene can be inserted into a suitable vector by methods well-known to those skilled in the art. For example, as a vector for Escherichia coli, pMW118 (manufactured by Nippon Gene Co., Ltd.), pUC18 (manufactured by TAKARA BIO), pKK233-2 (manufactured by Amersham-Pharmacia-Biotech), pET3d (manufactured by STRATAGENE) and the like, can be used and, as a vector for Bacillus subtilis, pUB110 (which can be purchased from American Type Culture Collection), and pHY300PLK (manufactured by TAKARABIO) and the like, can be used.

For example, when a gene is amplified using PCR primers 1 and 2, a plasmid having a sequence shown in Fig.2 can be selected by inserting the amplified gene into plasmid pMW118 which has been cut with SmaI in advance. This is used to transform, for example, Escherichia coli TG-1, an ampicillin resistant strain is then selected, and the resulting recombinant plasmid-harboring strain is cultured, and by extracting a plasmid, a GP gene can thereby be obtained.

(2. Improving the thermostability of α-glucan phosphorylase)

A method according to the present invention includes modifying a first nucleic acid molecule comprising a base sequence encoding a first α-glucan phosphorylase to obtain a second nucleic acid molecule containing a modified base sequence; preparing an expression vector comprising the second nucleic acid molecule; introducing the expression vector into a cell, to express an α-glucan phosphorylase having improved thermostability; and recovering the

expressed α-glucan phosphorylase having improved thermostability.

(2.1 Isolation of nucleic acid molecule comprising base sequence encoding first (natural) α-glucan phosphorylase)

A nucleic acid molecule comprising a base sequence encoding α-glucan phosphorylase having improved thermostability according to the present invention is also within the scope of the present invention. Such a nucleic acid molecule can be obtained by using methods known in the art, based on the disclosure of the present specification.

A nucleic acid molecule comprising a base sequence encoding natural α-glucan phosphorylase can be isolated directly from a plant producing a naturally occurring α-glucan phosphorylase, as described above.

For example, firstly, natural α-glucan phosphorylase is isolated from potato, Arabidopsis thaliana, spinach or the like. To exemplify a procedure for potato-derived α-glucan phosphorylase, firstly, 1.4kg of commercially available potato tubers are peeled. The tuber, a skin of which has been removed, is mashed in a juicer to obtain a fluid mash. Then, this fluid mash is filtered with a gauge to obtain a filtrate. To the filtrate is added a Tris buffer (pH 7.0) to a final concentration of 100 mM, to obtain an enzyme solution. This enzyme solution is further heated in a water bath at 55°C for further 10 minutes, after which the liquid temperature reaches 50°C. After heating, this enzyme solution is centrifuged at 8,500 rpm for 20 minutes using a centrifuge (AVANTI J-25I manufactured by BECKMANN) to remove insoluble proteins, and thus obtaining a supernatant.

Ammonium sulfate is added to the supernatant to a final concentration of 100 g/L, and this is allowed to stand at 4°C for 2 hours to precipitate proteins. Then, a centrifuge (AVANTI J-25I manufactured by BECKMANN) is used to centrifuge the solution at 8,500 rpm for 20 minutes, to remove insoluble proteins. Further, ammonium sulfate is added to the resulting supernatant to a final concentration of 250g/L, and this is allowed to stand at 4°C for 2 hours to precipitate proteins. Then, a centrifuge (AVANTI J-25I manufactured by BECKMANN) is used to centrifuge the solution at 8,500 rpm for 20 minutes, to recover insoluble proteins.

The recovered insoluble proteins are suspended in 150ml of 25 mM Tris buffer (pH 7.0). The suspended enzyme solution is dialyzed overnight against the same buffer. The sample after dialysis is adsorbed onto an anion exchange resin Q-Sepharose (manufactured by Pharmacia) which has been pre-equilibrated, and they washed with a buffer containing 200 mM sodium chloride. Subsequently, the proteins are eluted with a buffer containing 400 mM sodium chloride, and the eluate is recovered to obtain a partially purified potato tuber-derived glucan phosphorylase-containing solution.

Depending on the purchased potato, an α-glucan phosphorylase-containing solution obtained at this stage, can be used in trypsin treatment, but further purification is necessary in some cases. In such cases, if necessary, the purified potato α-glucan phosphorylase-containing solution can be obtained by combining a fraction from gel filtration chromatography using, for example, Sephacryl S-200HR (manufactured by Pharmacia), and a fraction from hydrophobic chromatography using, for example,

Phenyl-TOYOPEARL650M (manufactured by Tosoh Corporation). Purification of α-glucan phosphorylase from other plant species can be performed similarly.

The thus obtained purified α-glucan phosphorylase is treated with trypsin, the resulting trypsin treated fragment is separated by HPLC, and the amino acid sequence of the N-terminus of each of the separated peptide fragments is determined using a peptide sequencer. Then, using synthetic oligonucleotide probes prepared based on the identified amino acid sequence, a suitable genome library or a cDNA library is screened, thereby, a nucleic acid molecule (also referred to as a gene) comprising a base sequence encoding natural α-glucan phosphorylase can be obtained. Fundamental strategies for preparing oligonucleotide probes and a DNA library, and screening them by hybridization of nucleic acids, are well-known to those skilled in the art. For example, see Sambrook, et al., Molecular Cloning: A Laboratory Manual (1989); DNA Cloning, vol. I and II (edited by D. N. Glover, 1985); Oligonucleotide Synthesis (edited by M. J. Gait, 1984); Nucleic Acid Hybridization (edited by B. D. Hames & S. J. Higgins, 1984).

Alternatively, based on homology to a base sequence of certain α-glucan phosphorylases for which a base sequence encoding α-glucan phosphorylase is known, for example, a cDNA library or a genome library is screened by hybridization using nucleic acid probes containing at least a part of this base sequence, thereby, a nucleic acid molecule containing the base sequence of another kind of α-glucan phosphorylase may be acquired. Such methods are known in the art.

Alternatively, degenerate primers corresponding to a

region which is conserved in the amino acid sequence of various α-glucan phosphorylases are prepared, and PCR is performed using, for example, a cDNA library or a genome library of an objective species as a template, a base sequence of α-glucan phosphorylase derived from the species may be acquired. Such methods are known in the art.

When a genome library or a cDNA library is screened, the resulting nucleic acid molecule can be subcloned using methods well-known to the person skilled in the art. For example, by mixing λ phage containing an objective gene, suitable Escherichia coli and suitable helper phage, a plasmid containing an objective gene can be easily obtained. Thereafter, by transforming suitable Escherichia coli using a solution containing a plasmid, an objective gene can be subcloned. By culturing the resulting transformants, a plasmid DNA may be obtained, for example, by an alkaline SDS method, and the base sequence of an objective gene can be determined. A method of determining a base sequence is well-known to those skilled in the art. Further, by using PCR primers synthesized based on a base sequence of a DNA fragment, and using a polymerase chain reaction (PCR) employing the genomic DNA or the cDNA of potato as a template, an α-glucan phosphorylase gene may be directly amplified .

In the present specification, the "nucleic acid molecule" may consist only of natural nucleotides, may contain non-natural nucleotides, or may consist of only non-natural nucleotides. Examples of a non-natural nucleotide include derivatized nucleotides (also refers to as nucleotide analogs). The "derivatized nucleotide" and the "nucleotide analog" refer to those nucleotides which are different from naturally occurring nucleotides, but have a similar function

to that of the original nucleotide. Such derivatized nucleotides and nucleotide analogs are well-known in the art. Examples of such derivatized nucleotides and nucleotide analogs include, but are not limited to phosphorothioate, phosphoramidate, methylphosphonate, chiral methylphosphonate, 2-O-methylribonucleotide, and peptide-nucleic acid (PNA).

(2.2 Modification of the first nucleic acid molecule comprising a base sequence encoding first α-glucan phosphorylase)

A first nucleic acid molecule comprising a base sequence encoding a first α-glucan phosphorylase is modified to obtain a second nucleic acid containing a modified base sequence. A first nucleic acid molecule can be a nucleic acid molecule having a base sequence encoding a natural α-glucan phosphorylase, obtained as in the above (2.1). The first nucleic acid molecule may also be a nucleic acid molecule comprising a base sequence encoding α-glucan phosphorylase which has substantially the same enzyme activity as the enzyme activity of natural α-glucan phosphorylase, and in which 1 or a few or more amino acids are substituted, deleted or added to a base sequence encoding natural α-glucan phosphorylase. The "has substantially the same enzyme activity" refers to the enzyme activity when α-glucan phosphorylase after modification is measured under the same conditions as that of α-glucan phosphorylase before modification is within ±20%, preferably within ±10%, more preferably within ±5% of enzyme activity of α-glucan phosphorylase before modification.

Modification can be performed by carrying out

site-directed mutagenesis, mutagenesis using a mutagen (treatment of a subject gene with a mutagenic agent such as nitrite, ultraviolet-ray treatment), or error prone PCR. It is preferable to use site-directed mutagenesis from the viewpoint that the objective mutation is easily obtained, because the objective modification can be introduced at an object site when site-directed mutagenesis is used. Alternatively, a nucleic acid molecule having an objective sequence may be directly synthesized. Such chemical synthesis methods are well-known in the art.

The present inventors found out that, by substituting an amino acid residue at a particular position in the amino acid sequence of a natural α-glucan phosphorylase derived from a plant, with another amino acid residue, the thermostability of the resulting α-glucan phosphorylase is improved. Such a particular position can be determined by aligning any of the following motif sequences, or the amino acid sequence of SEQ ID NO: 2, and a comparison subject amino acid sequence:

motif sequence 1L: H-A-E-F-T-P-V-F-S (SEQ ID NO: 44) or a motif sequence 1H: H-A-Q-Y-S-P-H-F-S (SEQ ID NO: 45),

motif sequence 2: A-L-G-N-G-G-L-G (SEW ID NO: 46), and

motif sequence 3L: R-I-V-K-F-I-T-D-V (SEQ ID NO: 47) or motif sequence 3H: R-I-V-K-L-V-N-D-V (SEQ ID NO: 48).

The motif sequences 1L, 2 and 3L are present in the amino acid sequence (SEQ ID NO: 2) of potato-derived type L α-glucan phosphorylase. These motif sequences are present in the following positions in potato type L α-glucan phosphorylase: motif sequence 1L: position 36 to position 44 of the amino acid sequence set forth in SEQ ID NO: 2; motif sequence 2: position 132 to 1position 39 of the amino acid sequence set

forth in SEQ ID NO: 2; motif sequence 3L: position 700 to position 708 of the amino acid sequence set forth in SEQ ID NO: 2. The motif sequences 1H, 2 and 3H are present in the amino acid sequence of rice-derived type H α-glucan phosphorylase. These motif sequences are present in the following positions in rice type H α-glucan phosphorylase: motif sequence 1H: position 36 to position 44 of the amino acid sequence set forth in SEQ ID NO: 24; motif sequence 2: position 132 to position 139 of the amino acid sequence set forth in SEQ ID NO: 24; motif sequence 3H: position 625 to position 633 of the amino acid sequence set forth in SEQ ID NO: 24. Generally, natural α-glucan phosphorylase has these motif sequences, or sequences having high homology to them. The position of these motif sequences in other plant-derived α-glucan phosphorylases can be easily determined by those skilled in the art.

In the method according to the present invention, a nucleic acid molecule comprising a base sequence encoding a first α-glucan phosphorylase is modified so that α-glucan phosphorylase having improved thermostability, encoded by a modified nucleic acid molecule having an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least one position selected from the group consisting of: a position corresponding to phenylalanine at position 342 (F39); a position corresponding to asparagine at position 135 (N135); and a position corresponding to threonine at position 706 (T706) in an amino acid sequence of SEQ ID NO: 2. Preferably, a nucleic acid molecule comprising a base sequence encoding the first α-glucan phosphorylase is modified so that an amino acid sequence at a position corresponding to phenylalanine at position 309 (F39) or a position corresponding to threonine

at position 706 (T706) of an amino acid sequence set forth in SEQ ID NO: 2, of α-glucan phosphorylase having improved thermostability, encoded by a modified nucleic acid molecule is different from that of the natural α-glucan phosphorylase.

The "position corresponding to phenylalanine at position 39 (P39) of an amino acid sequence of SEQ ID NO: 2" as used in the present specification refers to a position which is aligned with phenylalanine at position 39 as set forth in SEQ ID NO: 2, when a subject amino acid sequence and an amino acid sequence of SEQ ID NO: 2 are aligned so that homology between the two sequences is highest, if necessary, by inserting a gap into one of sequences. When a gap is introduced into SEQ ID NO: 2, the gap is not counted when calculating the number of amino acid residues. More preferably, the above phrase refers to position which is aligned with phenylalanine at position 39 of SEQ ID NO: 2 when an amino acid sequence of SEQ ID NO: 2 and a subject amino acid sequence are aligned under the condition of GAP Penalty (Peptide): Insert=-10, Extend=-3, gap Extend on top position: setted (checked), Match Mode: Local Match using a score table of default, in multiple alignment of GENETYX-WIN Ver.4.0. A score table of default with respect to an amino acid is shown in the following Table 3.

(Table 3)

```
C  12,
S   0,  2,
T  -2,  1,  3,
P  -3,  1,  0,  6,
A  -2,  1,  1,  1,  2,
G  -3,  1,  0, -1,  1,  5,
N  -4,  1,  0, -1,  0,  0,  2,
D  -5,  0,  0, -1,  0,  1,  2,  4,
E  -5,  0,  0, -1,  0,  0,  1,  3,  4,
Q  -5, -1, -1,  0,  0, -1,  1,  2,  2,  4,
H  -3, -1, -1,  0, -1, -2,  2,  1,  1,  3,  6,
R  -4,  0, -1,  0, -2, -3,  0, -1, -1,  1,  2,  6,
K  -5,  0,  0, -1, -1, -2,  1,  0,  0,  1,  0,  3,  5,
M  -5, -2, -1, -2, -1, -3, -2, -3, -2, -1, -2,  0,  0,  6,
I  -2, -1,  0, -2, -1, -3, -2, -2, -2, -2, -2, -2, -2,  2,  5,
L  -6, -3, -2, -3, -2, -4, -3, -4, -3, -2, -2, -3, -3,  4,  2,  6,
V  -2, -1,  0, -1,  0, -1, -2, -2, -2, -2, -2, -2, -2,  2,  4,  2,  4,
F  -4, -3, -3, -5, -4, -5, -4, -6, -5, -5, -2, -4, -5,  0,  1,  2, -1,  9,
Y   0, -3, -3, -5, -3, -5, -2, -4, -4, -4,  0, -4, -4, -2, -1, -1, -2,  7, 10,
W  -8, -2, -5, -6, -6, -7, -4, -7, -7, -5, -3,  2, -3, -4, -5, -2, -6,  0,  0, 17,
B  -4,  0,  0, -1,  0,  0,  2,  3,  2,  1,  1, -1,  1, -2, -2, -3, -2, -5, -3, -5,  2,
Z  -5,  0, -1,  0,  0, -1,  1,  3,  3,  3,  2,  0,  0, -2, -2, -3, -2, -5, -4, -6,  2,  3,
X   0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0,  0
    C  S  T  P  A  G  N  D  E  Q  H  R  K  M  I  L  V  F  Y  W  B  Z  X
```

The multiple alignment of GENETYX-WIN Ver.4.0 is based on the following algorithm. In this alignment program, all possible pairs of sequences are aligned, two sequence alignment is performed as a round robin (pair wise alignment) and, among that, sequences of a combination having a high conservation ratio (score in pair wise alignment) are determined as common sequences, a hypothetical sequence is produced from common sequences (a common part remains as it is and, with respect to non-common parts, any one of the sequences is selected). A round robin between all sequences except for the sequence constituting the hypothetical sequence, and a hypothetical sequence is generated by the

same procedure until the final hypothetical sequence is produced. Thereafter, by applying information on insertion and shift of GAP used to produce the hypothetical sequence, to the original sequence, to constitute a whole, and the multiple alignment is completed. A calculation equation for this pair wise alignment is as follows.

When sequences a and b, each having a sequence length of m or n, and respective sequences are expressed as:

a=a1 a2 a3 … am

b= b1 b2 b3 … bm,

a GAP penalty g is indicated by the following equation:

$-g=s(ai,\phi)=a(\phi,bj)$.

An equation for obtaining an alignment score is as follows:

$G(0,0)=0$

$G(i,0)=i(-g)$

$G(0,j)=j(-g)$

$-gk=-[\alpha+\beta\ (k-1)]$

$E(i,j)=\{G(i-1,j)-\ \alpha,E(i-1,j)-\ \beta\}$

$F(i,j)=\max\{G(i,j-1)-\ \alpha,F(i,j-1)-\ \beta\}$

$G(i,j)=\max\{E(i,j),G(i-1,j-1)+s(ai,bj),F(i,j)\}$

α is the GAP insertion penalty, and β is the GAP extension penalty. E, F and G are a score matrix and, based on this, a pass matrix is produced.

A position corresponding to asparagine at position 135 (N135) and a position corresponding to threonine at position 706 (T706) are similarly construed.

In multiple alignments of GENETYX-WIN Ver.4.0, under

the aforementioned condition, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, and SEQ ID NO: 30 were aligned with SEQ ID NO: 2. As a result, phenylalanine or tyrosine was aligned at a position corresponding to phenylalanine at position 39 (F39) of the amino acid sequence set forth in SEQ ID NO: 2, asparagine was aligned at a position corresponding to asparagine at position 135 (N135) of an amino acid sequence of SEQ ID NO: 2, and threonine, asparagine or aspartic acid was aligned at a position corresponding to threonine at position 706 (T706) of the amino acid sequence set forth in SEQ ID NO: 2. Results of this alignment are shown in Fig. 1A to Fig. 1I. In Fig. 1A to Fig. 1I, "potato type L" represents the amino acid sequence (SEQ ID NO: 2) of a potato-derived type L α-glucan phosphorylase. "Potato type L2" represents the amino acid sequence (SEQ ID NO: 6) of a potato-derived second type L α-glucan phosphorylase. "Sweet potato type L" represents the amino acid sequence (SEQ ID NO: 4) of a sweet potato-derived type L α-glucan phosphorylase. "Fava bean type L" represents the amino acid sequence (SEQ ID NO: 8) of a Fava bean-derived type L α-glucan phosphorylase. "Arabidopsis thaliana type L" represents the amino acid sequence (SEQ ID NO: 10) of Arabidopsis thaliana-derived type L α-glucan phosphorylase. "Spinach" represents the amino acid sequence (SEQ ID NO: 12) of a spinach-derived type L α-glucan phosphorylase. "Rice type L" represents the amino acid sequence (SEQ ID NO: 16) of a rice-derived type L α-glucan phosphorylase. "Rice type L2" represents the amino acid sequence (SEQ ID NO: 18) of a rice-derived second type L α-glucan phosphorylase. "Corn type L" represents the amino acid sequence (SEQ ID NO: 14) of a corn-derived type L α-glucan phosphorylase. "Potato

type H" represents the amino acid sequence (SEQ ID NO: 30) of a potato-derived type H α-glucan phosphorylase. "Fava bean type H" represents the amino acid sequence (SEQ ID NO: 26) of a Fava bean-derived type H α-glucan phosphorylase. "Arabidopsis thaliana type H" represents the amino acid sequence (SEQ ID NO: 28) of an Arabidopsis thaliana-derived type H α-glucan phosphorylase. "Rice type H" represents the amino acid sequence (SEQ ID NO: 24) of a rice-derived type H α-glucan phosphorylase. "Wheat" represents the amino acid sequence (SEQ ID NO: 20) of a wheat-derived type H α-glucan phosphorylase. "Citrus type H" represents the amino acid sequence (SEQ ID NO: 22) of a Citrus hybrid cultivar-derived type H α-glucan phosphorylase. "E. coli MalQ" represents the amino acid sequence (SEQ ID NO: 35) of an Escherichia coli-derived maltodextrin phosphorylase. Maltodextrin phosphorylase is one kind of α-glucan phosphorylases.

For example, in sweet potato-derived type L α-glucan phosphorylase, a position corresponding to phenylalanine at position 39 (F39) of an amino acid sequence of SEQ ID NO: 2 is position 39 of an amino acid sequence of SEQ ID NO: 4, position corresponding to asparagine at position 135 (N135) of the amino acid sequence set forth in SEQ ID NO: 2 is position 135 of an amino acid sequence set forth in SEQ ID NO: 4, and a position corresponding to threonine at position 706 (T706) of the amino acid sequence set forth in SEQ ID NO: 2 is position 702 of the amino acid sequence set forth in SEQ ID NO: 4.

For example, in potato-derived second type L α-glucan phosphorylase, a position corresponding to F39 of an amino acid sequence set forth in SEQ ID NO: 2 is position 11 of the amino acid sequence set forth in SEQ ID NO: 6, a position

corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 107 of the amino acid sequence set forth in SEQ ID NO: 6, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 683 of the amino acid sequence set forth in SEQ ID NO: 6.

For example, in Fava bean-derived type L α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 43 of the amino acid sequence set forth in SEQ ID NO: 8, a position corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 139 of the amino acid sequence set forth in SEQ ID NO: 8, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 729 of the amino acid sequence set forth in SEQ ID NO: 8.

For example, in Arabidopsis thaliana-derived type L α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 106 of the amino acid sequence set forth in SEQ ID NO: 10, a position corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 202 of the amino acid sequence set forth in SEQ ID NO: 10, and the position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 752 of the amino acid sequence set forth in SEQ ID NO: 10.

For example, in spinach-derived type L α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 112 of the amino acid sequence set forth in SEQ ID NO: 12, a position

corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 208 of the amino acid sequence set forth in SEQ ID NO: 12, and a position corresponding to T706 of the amino acid sequence of SEQ ID NO: 2 is position 761 of the amino acid sequence set forth in SEQ ID NO: 12.

For example, in corn-derived type L α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 95 of the amino acid sequence set forth in SEQ ID NO: 14, a position corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 191 of the amino acid sequence set forth in SEQ ID NO: 14, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 773 of the amino acid sequence set forth in SEQ ID NO: 14.

For example, in rice-derived type L α-glucan phosphorylase, a position corresponding to F39 of an amino acid sequence of SEQ ID NO: 2 is position 41 of the amino acid sequence set forth in SEQ ID NO: 16, a position corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 137 of the amino acid sequence set forth in SEQ ID NO: 16, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 718 of the amino acid sequence set forth in SEQ ID NO: 16.

For example, in another rice-derived type L α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 91 of the amino acid sequence set forth in SEQ ID NO: 18, a position corresponding to N135 of the amino acid sequence set forth

in SEQ ID NO: 2 is position 187 of the amino acid sequence set forth in SEQ ID NO: 18, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 741 of the amino acid sequence set forth in SEQ ID NO: 18.

For example, in wheat-derived type H α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 31 of the amino acid sequence set forth in SEQ ID NO: 20, a position corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 127 of the amino acid sequence set forth in SEQ ID NO: 20, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is a position 622 of the amino acid sequence set forth in SEQ ID NO: 20.

For example, in a Citrus hybrid cultivar-derived type H α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 42 of the amino acid sequence set forth in SEQ ID NO: 22, a position corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is a position 138 of the amino acid sequence set forth in SEQ ID NO: 22, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 630 of the amino acid sequence set forth in SEQ ID NO: 22.

For example, in rice-derived type H α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 39 of the amino acid sequence set forth in SEQ ID NO: 24, a position corresponding to N135 of the amino acid sequence set forth

in SEQ ID NO: 2 is position 135 of set forth in amino acid sequence set forth inEQ ID NO: 24, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 631 of the amino acid sequence set forth in SEQ ID NO: 24.

For example, in Fava bean-derived type H α-glucan phosphorylase, a position corresponding to F39 of an amino acid sequence of SEQ ID NO: 2 is position 43 of the amino acid sequence set forth in SEQ ID NO: 26, a position corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 139 of the amino acid sequence set forth in SEQ ID NO: 26, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 632 of the amino acid sequence set forth in SEQ ID NO: 26.

For example, in Arabidopsis thaliana-derived type H α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 40 of the amino acid sequence set forth in SEQ ID NO: 28, a position corresponding to N135 of the amino acid sequence set forth in SEQ ID NO: 2 is position 136 of the amino acid sequence set forth in SEQ ID NO: 28, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 631 of the amino acid sequence set forth in SEQ ID NO: 28.

For example, in potato-derived type H α-glucan phosphorylase, a position corresponding to F39 of the amino acid sequence set forth in SEQ ID NO: 2 is position 36 of the amino acid sequence set forth in SEQ ID NO: 30, a position corresponding to N135 of the amino acid sequence set forth

in SEQ ID NO: 2 is position 133 of the amino acid sequence set forth in SEQ ID NO: 30, and a position corresponding to T706 of the amino acid sequence set forth in SEQ ID NO: 2 is position 628 of the amino acid sequence set forth in SEQ ID NO: 30.

A position of an amino acid residue which improves thermostability can be determined by not only alignment with the sequences 916 amino acid residues set forth in SEQ ID NO: 2, but also by alignment with one or more sequences selected from the group consisting of the aforementioned motif sequences 1L or 1H, 2, and 3L or 3H. As far as the heretofore known plant-derived α-glucan phosphorylases were aligned, the thus determined position is the same in either the case where SEQ ID NO: 2 is used and the case where motif sequences 1L or 1H, 2, and 3L or 3H are used.

The motif sequence 1L is well conserved in type L α-glucan phosphorylases, while the motif sequence 1H is well conserved in type H α-glucan phosphorylase. It can be said that a position corresponding to phenylalanine at position 39 (F39) of the amino acid sequence set forth in SEQ ID NO: 2 is a position corresponding to position 4 in the motif sequence 1L or 1H.

The motif sequence 2 is commonly conserved in type L and type H α-glucan phosphorylases. It can be said that a position corresponding to asparagine at position 135 (N135) of the amino acid sequence set forth in SEQ ID NO: 2 is a position corresponding to position 4 in the motif sequence 2.

The motif sequence 3L is well conserved in type L α-glucan

phosphorylases, while the motif sequence 3H is well conserved in type H α-glucan phosphorylases. It can be said that a position corresponding to threonine at position 706 (T706) of the amino acid sequence set forth in SEQ ID NO: 2 is a position corresponding to position 7 in the motif sequence 3L or 3H.

In this manner, the position of an amino acid residue which improves thermostability can be also specified using the motif sequences. A position of an amino acid residue which improves thermostability can be at least one position selected from the group consisting of a position corresponding to position 4 in a motif sequence 1L: H-A-E-F-T-P-V-F-S or a position corresponding to position 4 in a motif sequence 1H: H-A-Q-Y-S-P-H-F-S; a position corresponding to position 4 in a motif sequence 2: A-L-G-N-G-G-L-G; and a position corresponding to position 7 in a motif sequence 3L: R-I-V-K-F-I-T-D-V or a position corresponding to position 7 in a motif sequence 3H: R-I-V-K-L-V-N-D-V.

Therefore, in the method according to the present invention, it can be said that a nucleic acid molecule comprising a base sequence encoding first α-glucan phosphorylase is modified so that α-glucan phosphorylase having improved thermostability, encoded by a modified nucleic acid has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least one position selected from the group consisting of a position corresponding to position 4 in a motif sequence 1L: H-A-E-F-T-P-V-F-S or a position corresponding to position 4 in a motif sequence 1H: H-A-Q-Y-S-P-H-F-S; a position corresponding to position 4

in a motif sequence 2: A-L-G-N-G-G-L-G; and a position corresponding to position 7 in a motif sequence 3L: R-I-V-K-F-I-T-D-V or a position corresponding to position 7 in a motif sequence 3H: R-I-V-K-L-V-N-D-V.

In the present specification, the "motif sequence" refers to a partial sequence which is seen between amino acid sequences of a plurality of proteins, and is commonly or highly conserved. Generally, the motif sequence has particular function in many cases, but in the present specification, even whena particular function is not identified, as long as the sequences is conserved between a plurality of amino acid sequences, this is called motif sequence.

An amino acid residue "at position 4 in a motif sequence 1L" refers to an amino acid residue which is fourth when counted in order, when the amino acid residue at an N-terminus (left end) of the motif sequence 1L is taken to be position 1. "position 4 in a motif sequence 1H", "position 4 in a motif sequence 2", "position 7 in a motif sequence 3L", "position 7 in a motif sequence 3H", and the like are similar.

These motif sequences are generally well conserved in plant α-glucan phosphorylases. The motif sequences 1L or 1H and 3L or 3H are well conserved in α-glucan plant phosphorylases, but are not conserved in α-glucan phosphorylases derived from animals, microorganisms, or the like. Motif sequence 2 is well conserved in α-glucan phosphorylases of almost all organisms such as plants, animals and microorganisms. The motif sequence 2 contains an amino acid residue which is presumed to be involved in binding of a substrate and binding of pyridoxal 5'-phosphate

which is a coenzyme, and is a part of the regions essential for activity. The positions of motif sequences 1L and 1H, and the position of the motif sequence 2 are shown in Fig. 1B. The positions of motif sequences 3L and 3H are shown in Fig. 1G.

As used herein, "a position corresponding to position 4 in a motif sequence 1L: H-A-E-F-T-P-V-F-S or a position corresponding to position 4 in a motif sequence 1H: H-A-Q-Y-S-P-H-F-S" refers to position which is aligned with amino acid residue at position 4 in the motif sequence 1L or the motif sequence 1H when a subject amino acid sequence and the motif sequence 1L or the motif sequence 1H are aligned, without inserting a gap, so that homology between sequences is greatest. More preferably, it refers to the position which is aligned with the amino acid residue at position 4 in the motif sequence 1L or the motif sequence 1H when maximum matching of GENETYX-WIN Ver.4.0 (Genetics Co., Ltd.) is performed under no gap condition.

A position corresponding to position 4 in motif sequence 2, and a position corresponding to position 7 in motif sequence 3L and a position corresponding to position 7 in motif sequence 3H are similarly construed.

Maximum matching of GENETYX-WIN Ver.4.0 is as follows: while substitution and deletion are considered, sequence data to be analyzed, and sequence data to be compared are aligned so that amino acid pairs matching between these sequences become greatest, thereupon, Matches, Mismatches, and Gaps are scored, respectively, a sum is calculated, and alignment at the lowest sum is outputted (Reference: Takashi, K., and Gotoh, O.1984. Sequence Relationships among Various

4.5 S RNA Species J.Biochem. 92:1173-1177). Preferably, alignment is performed under the condition of Matches=-1; Mismatches=1; Gaps=None; *N+=2.

Using maximum matching of GENETYX-WIN Ver.4.0, potato type L (SEQ ID NO: 2), sweet potato type L (SEQ ID NO: 4), potato second type L (SEQ ID NO: 6), Fava bean type L (SEQ ID NO: 8), Arabidopsis thaliana type L (SEQ ID NO: 10), spinach type L (SEQ ID NO: 12), corn type L (SEQ ID NO: 14), rice type L (SEQ ID NO: 16), rice second type L (SEQ ID NO: 18), wheat type H (SEQ ID NO: 20), Citrus hybrid cultivar type H (SEQ ID NO: 22), rice type H (SEQ ID NO: 24), Fava bean type H (SEQ ID NO: 26), Arabidopsis thaliana type H (SEQ ID NO: 28) and potato type H (SEQ ID NO: 30) were aligned with a motif sequence 1L or a motif sequence 1H. Analysis of maximum matching was performed under the condition of Matches=-1; Mismatches=1; Gaps=0; *N+=2.

In maximum matching of GENETYX-WIN Ver.4.0, under the aforementioned condition, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, and SEQ ID NO: 30 were aligned with each motif sequence (motif sequence 1L, 1H, 2, 3L or 3H). As a result, phenylalanine or tyrosine was aligned with a position corresponding to position 4 in the motif sequence 1L or a position corresponding to position 4 in the motif sequence 1H, asparagine was aligned with a position corresponding to position 4 in the motif sequence 2, and threonine, asparagine or aspartic acid was aligned with a position corresponding to position 7 in the motif sequence 3L or a position corresponding to position 7 in the motif sequence 3H. The motif sequences 1L, 2 and 3L are

partial sequences of SEQ ID NO: 2, and motif sequences 1H, 2 and 3H are partial sequences of SEQ ID NO: 24.

Regarding each of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, and SEQ ID NO: 30, the results of an alignment using a full length of SEQ ID NO: 2, and results of an alignment using motif sequences 1L, 1H, 2, 3L and 3H were compared. As a result, in each of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, and SEQ ID NO: 30, a position corresponding to position 39 of SEQ ID NO: 2, and a position corresponding to position 4 in the motif sequence 1L or 1H were the same. A position corresponding to position 135 of SEQ ID NO: 2, and a position corresponding to position 4 in the motif 2 were the same. A position corresponding to position 706 of SEQ ID NO: 2, and a position corresponding to position 7 in the motif 3L or 3H were the same. In this manner, it was confirmed that, even when alignment was performed using motif sequences, the same positions are specified as those specified when the amino acid sequence of SEQ ID NO: 2 is used.

A nucleic acid molecule comprising a modified base sequence obtained by modifying a nucleic acid molecule comprising a base sequence encoding the amino acid sequence represented in position 1 to position 916 of SEQ ID NO: 2, position 1 to position 912 of SEQ ID NO: 4, position 1 to position 893 of SEQ ID NO: 6, position 1 to position 939 of SEQ ID NO: 8, position 1 to position 962 of SEQ ID NO:

10, position 1 to position 971 of SEQ ID NO: 12, position 1 to position 983 of SEQ ID NO: 14, position 1 to position 928 of SEQ ID NO: 16, position 1 to position 951 of SEQ ID NO: 18, position 1 to position 832 of SEQ ID NO: 20, position 1 to position 840 of SEQ ID NO: 22, position 1 to position 841 of SEQ ID NO: 24, position 1 to position 842 of SEQ ID NO: 26, position 1 to position 841 of SEQ ID NO: 28, and position 1 to position 838 of SEQ ID NO: 3 set forth in the Sequence Listing is within the scope of the present invention.

A nucleic acid molecule comprising a modified base sequence obtained by modifying a nucleic acid molecule comprising a base sequence represented by SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, or SEQ ID NO: 29 set forth in the Sequence Listing is within the scope of the present invention.

A nucleic acid molecule comprising a modified base sequence obtained by modifying a nucleic acid molecule comprising a base sequence encoding an amino acid sequence having at least 50% identity with an amino acid sequence selected from the group consisting of: position 1 to position 916 of SEQ ID NO: 2; position 1 to position 912 of SEQ ID NO: 4; position 1 to position 893 of SEQ ID NO: 6; position 1 to position 939 of SEQ ID NO: 8; position 1 to position 962 of SEQ ID NO: 10; position 1 to position 971 of SEQ ID NO: 12; position 1 to position 983 of SEQ ID NO: 14; position 1 to position 928 of SEQ ID NO: 16; position 1 to position 951 of SEQ ID NO: 18; position 1 to position 832 of SEQ ID NO: 20; position 1 to position 840 of SEQ ID NO: 22; position 1 to position 841 of SEQ ID NO: 24; position 1 to position

842 of SEQ ID NO: 26; position 1 to position 841 of SEQ ID NO: 28; and position 1 to position 838 of SEQ ID NO: 30; as set forth in the Sequence Listing is within the scope of the present invention.

In the present invention, "identity" of a sequence such as an amino acid sequence and a base sequence refers to the degree of occurrence of the same amino acid (base when base sequences are compared) between two sequences. Identity is generally determined by comparing two amino acid sequences or two base sequences, and comparing these two sequences which are aligned in an optimal format, which can contain additions or deletions. Percentage identity is calculated by determining the number of positions where an amino acid (base when base sequences are compared) is the same between these two sequences, dividing the number of the same positions by a total number of compared positions, and multiplying the obtained result by 100 in order to obtain a percentage identity between the two sequences.

As an example, an amino acid sequence of natural α-glucan phosphorylase used for obtaining α-glucan phosphorylase having improved thermostability of the present invention may be the same as, that is, 100% identical with an amino acid sequence (i.e. control amino acid sequence) selected from the group consisting of position 1 to position 916 of SEQ ID NO: 2; position 1 to position 912 of SEQ ID NO: 4; position 1 to position 893 of SEQ ID NO: 6; position 1 to position 939 of SEQ ID NO: 8; position 1 to position 962 of SEQ ID NO: 10; position 1 to position 971 of SEQ ID NO: 12; position 1 to position 983 of SEQ ID NO: 14; position 1 to position 928 of SEQ ID NO: 16; position 1 to position 951 of SEQ ID NO: 18; position 1 to position 832 of SEQ ID

NO: 20; position 1 to position 840 of SEQ ID NO: 22; position 1 to position 841 of SEQ ID NO: 24; position 1 to position 842 of SEQ ID NO: 26; position 1 to position 841 of SEQ ID NO: 28; and position 1 to position 838 of SEQ ID NO: 30; or this amino acid sequence may one or more altered amino acid residues as compared with a control amino acid sequence. Such alterations can be selected from the group consisting of a deletion, a substitution including conservative and non-conservative substitution, or an insertion of at least one amino acid. This alteration may occur at a position of an amino terminus or a carboxyl terminus of a control amino acid sequence, or may occur at any position other than these terminuses. Alteration of an amino acid residue may be interspersed with one residue, or a few residues may be contiguous.

In the present specification, the percentage identity of sequences is calculated using maximum matching of GENETYX-WIN Ver.4.0 (Genetics Co., Ltd.). This program aligns sequence data to be analyzed, and sequence data to be compared so that amino acid pairs matched between sequences become greatest while substitution and deletion are considered, and thereupon, gives a score to each of Matches, Mismatches, and Gaps, calculates a sum, outputs alignment at the smallest sum, and calculates identity thereupon (Reference: Takashi, K., and Gotoh, O. 1984. Sequence Relationships among Various 4.5 S RNA Species J. Biochem. 92:1173-1177).

Using maximum matching of GENETYX-WIN Ver.4.0, the percentage identity of sweet potato type L (SEQ ID NO: 4), potato second type L (SEQ ID NO: 6), Fava bean type L (SEQ ID NO: 8), Arabidopsis thaliana type L (SEQ ID NO: 10), spinach

type L (SEQ ID NO: 12), corn type L (SEQ ID NO: 14), rice type L (SEQ ID NO: 16), rice second type L (SEQ ID NO: 18), and wheat type H (SEQ ID NO: 20), Citrus hybrid cultivar type H (SEQ ID NO: 22), rice type H (SEQ ID NO: 24), Fava bean type H (SEQ ID NO: 26), Arabidopsis thaliana type H (SEQ ID NO: 28) and potato type (SEQ ID NO: 30) with potato type L (SEQ ID NO: 2) was calculated, and results are shown in Table 4. Analysis of maximum matching was performed under the condition of Matches=-1; Mismatches=1; Gaps=1; *N+=2.

Table 4

Subject sequence	Identity
Potato type L	100
Potato second type L	70.3
Arabidopsis thaliana type L	72.1
Spinach type L	72.7
Rice type L	73.8
Rice second type L	67.7
Corn type L	70.2
Sweet potato type L	78.6
Fava bean type L	72.5
Potato type H	57.5
Arabidopsis thaliana type H	57.8
Rice type H	57.0
Fava bean type H	58.6
Citrus hybrid cultivar type H	57.5
Wheat type H	57.6

A nucleic acid molecule comprising a modified base sequence obtained by modifying a nucleic acid molecule which hybridizes under stringent condition with a nucleic acid molecule consisting of a base sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, and SEQ ID NO: 29 as set forth in the Sequence Listing is within

the scope of the present invention. Those skilled in the art can easily select a desired α-glucan phosphorylase gene.

As used herein, the term "stringent condition" refers to conditions under which a sequence hybridizes with a specific sequence, but not with a non-specific sequence. Selection of appropriate stringent conditions is well-known to those skilled in the art, and is described, for example, in Molecular Cloning (Sambrook, et al., supra). Specifically, the conditions mean that a polynucleotide which can be identified using the conditions under which hybridization is performed at 65°C in a solution containing 50% formamide, 5 ×SSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5×Denhart's solution (0.2% BSA, 0.2% Ficoll 400 and 0.2% polyvinylpyrrolidone), 10% dextran sulfate, and 20 μg/ml denatured sheared salmon sperm DNA using a filter on which a DNA derived from a colony or a plaque has been immobilized, and a filter is washed under the condition of 65°C using a SSC (saline-sodium citrate) solution having a 0.1 to 2-fold concentration (a composition of a SSC solution having a 1-fold concentration is 150 mM sodium chloride, 15 mM sodium citrate).

A modified nucleic acid molecule used in the method of the present invention may be a nucleic acid molecule which was conservatively modified relative to a nucleic acid molecule comprising a base sequence encoding a first α-glucan phosphorylase. The "nucleic acid molecule which was conservatively modified relative to a nucleic acid molecule comprising a base sequence encoding first α-glucan phosphorylase " refers to a nucleic acid molecule comprising a base sequence encoding an amino acid sequence which is the same or essentially the same as an amino acid sequence

encoded by a base sequence encoding the first α-glucan phosphorylase. The "amino acid sequence which is essentially the same as an amino acid sequence encoded by a base sequence encoding first α-glucan phosphorylase " refers to an amino acid sequence having essentially the same enzyme activity as that of first α-glucan phosphorylase. Due to degeneracy of a genetic code, many functionally equivalent base sequences encode a prescribed amino acid sequence. For example, codons GCA, GCC, GCG and GCT all encode the amino acid alanine. Therefore, at all positions where alanine is specified by a GCA codon, the codon can be changed to GCC, GCG or GCT without changing the encoded alanine. Similarly, regarding an amino acid encoded by a plurality of codons, at all positions where the amino acid is specified by a codon, the codon can be changed to any another codon encoding the amino acid without changing the particular amino acid coded. Such a variation in a base sequence is a "silent mutation" which is one kind of conservatively altered mutation. All base sequences in the present specification which encode a polypeptide also include all possible silent alterations of the nucleic acid. Silent mutation includes "silent substitution" in which a coding nucleic acid is not changed, and the case where a nucleic acid does not originally encode an amino acid. When a certain nucleic acid encodes an amino acid, silent mutation has the same meaning as that of silent substitution. In the present specification, "silent substitution" refers to substitution of a base sequence encoding a certain amino acid with another base sequence encoding the same amino acid, in a base sequence. Based on the phenomenon of degeneracy in a genetic code, in the case where there are a plurality of base sequences encoding a certain amino acid (e.g. glycine), such a silent substitution is possible. Therefore, a polypeptide having

an amino acid sequence encoded by a base sequence produced by silent substitution has the same amino acid sequence as that of the original polypeptide. Therefore, the α-glucan phosphorylase having improved thermostability of the present invention can include silent substitutions at a base sequence level, in addition to modification which is aimed a by the present invention (substitution is performed so that the α-glucan phosphorylase has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least one position selected from the group consisting of a position corresponding to position 4 in the motif sequence 1L or 1H, a position corresponding to position 4 in a motif sequence 2, or a position corresponding to position 7 in the motif sequence 3L or 3H, or a position corresponding to phenylalanine at position 39 (F39) in an amino acid sequence of SEQ ID NO: 2, a position corresponding to asparagine at position 135 (N135) and a position corresponding to threonine at position 706 (T706). In the art, it is understood that each codon in a nucleic acid (except for ATG which is only one codon usually encoding methionine, and TGG which is only one codon usually encoding tryptophan) can be modified in order to produce the functionally same molecule. Therefore, each silent mutation of a nucleic acid encoding a polypeptide is implicitly included in each described sequence. Preferably, such the alteration can be performed so that substitution of cysteine, which is an amino acid that greatly influences the conformation of a polypeptide, is avoided.

A base sequence encoding α-glucan phosphorylase having improved thermostability of the present invention can be changed in conformity with a codon usage in an organism into which the sequence is introduced for expression. Codon usage

reflects the usage in a gene which is highly expressed in the organism. For example, when expression is intended in Escherichia coli, the sequence can be made to be optimal for expression in Escherichia coli according to the published codon usage table (e.g. Sharp, et al., Nucleic Acids Research 16, No.17, p.8207 (1988)).

(2.3 Making expression vectors)

An expression vector is made using a nucleic acid molecule comprising the base sequence modified as described above. A method for preparing an expression vector using a particular nucleic acid sequence is well-known to those skilled in the art.

When a nucleic acid molecule is referred in the present specification, a "vector" refers to a nucleic acid molecule which can transfer an objective base sequence into an objective cell. Examples of such vectors include a vector which can autonomously replicate in an objective cell, or can be incorporated into a chromosome of an objective cell, and has a promoter at a position suitable for transcribing a modified base sequence. In the present specification, the vector may be a plasmid.

As used herein, the "expression vector" refers to a vector which can express a modified base sequence (i.e. base sequence encoding modified α-glucan phosphorylase) in an objective cell. An expression vector contains, in addition to a modified base sequence, various regulation elements such as a promoter regulating expression thereof and, if necessary, factors necessary for replication in an objective cell and selection of a recombinant (e.g. origin of replication (ori),

and a selectable marker such as a drug resistant gene). In an expression vector, a modified base sequence is operably linked so that it is transcribed and translated. Regulation elements include a promoter, a terminator and an enhancer. In addition, when secretion of an expressed enzyme outside a cell is intended, a base sequence encoding a secretion signal peptide is linked upstream of a modified base sequence in the correct reading frame. It is a matter well-known to those skilled in the art, that both the type of an expression vector used for introduction into a particular organism (e.g. bacterium), and the kind of a regulation element and other factors used in the expression vector, can vary depending on an objective cell.

As used herein, the "terminator" is a sequence which is situated downstream of a protein coding region, and is involved in termination of transcription upon transcription of a base sequence into an mRNA, and in the addition of a poly A sequence. It is known that the terminator influences the expression level of a gene with regard to the stability of an mRNA.

As used herein, the "promoter" refers to a region on a DNA which determines a transcription initiation site of a gene, and directly regulates the transcription frequency, and is a base sequence to which a RNA polymerase binds, thereby, initiating transcription. Since the region of a promoter is usually a region about 2 kbp or less upstream of a first exon of a putative protein coding region in many cases, when a protein coding region in a genome base sequence is predicted using a DNA analyzing software, a promoter region can be putative. A putative promoter region varies with every structural gene, and is usually upstream of a structural

gene without limitation, and may be downstream of a structural gene. Preferably, a putative promoter region is present about 2 kbp or less upstream of a first exon translation initiation point.

As used herein, the "enhancer" can be used for enhancing the expression efficiency of an objective gene. Such an enhancer is well-known in the art. A plurality of enhancers can be used, but only one may be used, or may not be used at all.

As used herein, "operably linked" refers to when a desired base sequence is placed under the control of a transcription and translation regulating sequence (e.g. promoter, enhancer and the like) or a translation regulating sequence which effect expression (i.e. operation). In order that a promoter is operably linked to a gene, usually, a promoter is disposed immediately upstream of the gene, but it is not necessary that a promoter is disposed adjacent to the gene.

In order to operably link a modified nucleic acid sequence to the aforementioned regulation element, an objective α-glucan phosphorylase gene should be processed in some cases. Examples include the case where the distance between a promoter and a coding region is too long, and reduction in a transcription efficiency is predicted, the case where the distance between a ribosome binding site and a translation initiation codon is not suitable, and the like. Examples of the procession means include digestion with a restriction enzyme, digestion with an exonuclease such as Bal31 and ExoIII, or introduction of site-directed mutagenesis using a single-stranded DNA such as M13 or PCR.

(2.4 Expression of α-glucan phosphorylase having improved thermostability)

Then, the expression vector prepared as described above is introduced into a cell, thereby, α-glucan phosphorylase having improved thermostability is expressed.

In the present specification, "expression" of an enzyme refers to in vivo or in vitro transcription and translation of a base sequence encoding the enzyme, and production of the encoded enzyme.

A cell into which an expression vector is introduced (also referred to as a host) includes prokaryotes and eukaryotes. A cell into which an expression vector is introduced can be easily selected, taking various conditions such as ease of expression of α-glucan phosphorylase, ease of culturing, growth rate, and safety into consideration. For example, when α-glucan phosphorylase is used in synthesizing amylose having a high molecular weight, since it is preferable that α-glucan phosphorylase does not contain amylase as a contaminant, it is preferable to use a cell which does not produce amylase or produce amylase only at a low level. Examples of such a cell include microorganisms such as bacteria and fungi. Examples of more preferable cells include mesophilic microorganisms (e.g. Escherichia coli, Bacillus subtilis). In the present specification, the "mesophilic microorganism" is a microorganism having a growth temperature in a normal temperature environment, and particularly refers to a microorganism having an optimal growth temperature of 20 °C to 40°C. A cell may be such as a microorganism cell, or may be a plant or animal cell. Depending on a cell to be used, an enzyme of the present

invention can be an enzyme which has undergone post-translational processing. A plant includes, but is not limited to, a dicot, and a monocot such as rice, wheat, barley and corn. A cereal such as rice has a nature of accumulating a storage protein in a seed and, using a storage protein system, the cereal can be expressed so that α-glucan phosphorylase having improved thermostability of the present invention is accumulated in a seed (see Japanese Laid-Open Publication No. 2002-58492 specification).

In the method of the present invention, the technique of introducing an expression vector into a cell may be any technique known in the art. Examples of such the technique include, for example, transformation, transduction, and transfection. Such the technique of introducing a nucleic acid molecule is well-known in the art, and is conventional, and described, for example, in Ausubel F.A., et al. ed. (1988), Current Protocols in Molecular Biology, Wiley, New York, NY; Sambrook J, et al. (1987) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, and Bessatsu Jikkenkagaku "Idenshidounyu & Hatsugen kaiseki jikkenhou", Yodosha, 1997.

When a plant cell is used as a cell, a method of re-differentiating a transformant into a tissue or a plant is well-known in the art. Examples of such a method are described in following: Rogers, et al., Methods in Enzymology 118:627-640 (1986); Tabata, et al., Plant Cell Physiol., 28:73-82 (1987); Shaw, Plant Molecular Biology: A Practical Approach. IRL press (1988); Shimamoto, et al., Nature 338: 274 (1989); and Maliga, et al., Methods in Plant Molecular Biology: A laboratory course. Cold Spring Harbor Laboratory Press (1995). A method of transforming a woody plant is

described in Molecular Biology of Woody Plants (Vol. I, II) (ed. S. Mohan Jain, Subhash C. Minocha), Kluwer Academic Publishers, (2000). In addition, a method of transforming a woody plant is described in detail, for example, in Plant Cell Reports (1999) 19:106-110. Therefore, those skilled in the art can re-differentiate a transformant by appropriately using the aforementioned well-known method depending on an objective transgenic plant. An objective gene is introduced in the thus obtained transgenic plant, and the introduction of a gene can be confirmed using the know method such as Northern blotting, and Western blot analysis or other well-known conventional techniques.

By culturing a cell into which an expression vector has been introduced, and has acquired the ability to express α-glucan phosphorylase having improved thermostability (also referred to as transformed cell), α-glucan phosphorylase having improved thermostability can be expressed in a cell. The condition of culturing a transformed cell is appropriately selected depending on a kind of a host cell to be used, and a kind of an expression regulating factor in an expression vector. For example, a usual shaking culture method can be used.

A medium used for culturing a transformed cell is not particularly limited as long as the cell used is grown, and can express objective α-glucan phosphorylase having improved thermostability. In a medium, in addition to a carbon source and a nitrogen source, inorganic salts such as salts of phosphoric acid, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Na^{+}, K^{+} and the like can be used alone, or by appropriately mixing them, if necessary. In addition, if necessary, various inorganic substances or organic substances necessary

for growing a transformed cell, or expressing objective α-glucan phosphorylase having improved thermostability may be added.

A temperature for culturing a transformed cell can be selected so as to be suitable for growing a transformed cell to be used. Usually, the temperature is 15°C to 60°C. Culturing of a transformed cell is continued for a sufficient time to express α-glucan phosphorylase having improved thermostability.

When an expression vector having an inducible promoter is used, expression can be controlled by addition of an inducer, change of a culturing temperature, and adjustment of medium components. For example, when an expression vector having a lactose inducible promoter is used, expression can be induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG).

(2.5 Recovery of α-glucan phosphorylase having improved thermostability)

The thus expressed α-glucan phosphorylase having improved thermostability can be then recovered. For example, when the expressed α-glucan phosphorylase having improved thermostability is produced in a transformed cell, a cell is recovered from a culture of transformed cells by centrifuging or filtering the culture. The recovered cell is suspended in a suitable buffer, and is crushed using a conventional means (ultrasound, French press, lysozyme treatment) to obtain a crude enzyme solution. Further, a crude enzyme solution or a purified enzyme having improved specific activity is obtained by purifying the crude enzyme

solution by a method of appropriately combining conventional enzyme purifying means such as centrifugation, chromatography, membrane fractionation, electrophoresis, and salting-out. When an enzyme hydrolyzing a glucan such as α-amylase is not contained, a crude enzyme as it is can be used, for example, in preparation of a glucan having a high-molecular weight.

By producing α-glucan phosphorylase having improved thermostability as described above, it becomes possible to considerably improve thermostability of natural α-glucan phosphorylase. In addition, the expressed α-glucan phosphorylase having improved thermostability can be simply purified utilizing the thermostability thereof. In brief, by heat-treating a cell extract containing α-glucan phosphorylase having improved thermostability at about 60°C, contaminating enzymes are insolubilized. By centrifuging the insolubilized substances to remove them, and performing dialysis treatment, purified α-glucan phosphorylase having improved thermostability is obtained.

(3. α-glucan phosphorylase having improved thermostability)

The α-glucan phosphorylase having improved thermostability according to the present invention obtained by the aforementioned method has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least one position selected from the group consisting of a position corresponding to position 4 in the motif sequence 1L: H-A-E-F-T-P-V-F-S or a position corresponding to position 4 in the motif sequence 1H: H-A-Q-Y-S-P-H-F-S; a position corresponding o position 4 in the motif sequence 2: A-L-G-N-G-G-L-G; and a position

corresponding to position 7 in the motif sequence 3L: R-I-V-K-F-I-T-D-V or a position corresponding to position 7 in the motif sequence 3H: R-I-V-K-L-V-N-D-V.

The α-glucan phosphorylase having improved thermostability according to the present invention has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least one position selected from the group consisting of a position corresponding to phenylalanine at position 39 (F39) of the amino acid sequence set forth in SEQ ID NO: 2, a position corresponding to asparagine at position 135 (N135) of the amino acid sequence set forth in SEQ ID NO: 2 and a position corresponding to threonine at position 706 (T706) of the amino acid sequence set forth in SEQ ID NO: 2. The α-glucan phosphorylase having improved thermostability of the present invention may contain an amino acid sequence in which ,in addition to substitution of an amino acid residue at these positions, one or a several amino acids are deleted, substituted or added relative to an amino acid sequence of natural α-glucan phosphorylase.

In one embodiment, the α-glucan phosphorylase having improved thermostability of the present invention contains an amino acid sequence in which one or a several amino acids are deleted, substituted or added relative to an amino acid sequence of plant-derived α-glucan phosphorylase, and has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least one position selected from the group consisting of a position corresponding to phenylalanine at position 39 (F39) of the amino acid sequence set forth in SEQ ID NO: 2, a position corresponding to asparagine at position 135 (N135) of the

amino acid sequence set forth in SEQ ID NO: 2 and a position corresponding to threonine at position 705 (T706) of the amino acid sequence set forth in SEQ ID NO: 2.

The enzyme of the present invention is α-glucan phosphorylase having improved thermostability, obtained by modifying plant-derived natural α-glucan phosphorylase, contains an amino acid sequence in which one or a several amino acids are deleted, substituted or added relative to an amino acid sequence of the natural α-glucan phosphorylase, and has an amino acid residue which is different from an amino acid residue of the natural α-glucan phosphorylase in at least one position selected from the group consisting of a position: corresponding to phenylalanine at position 39 (F39) of the amino acid sequence set forth in SEQ ID NO: 2; a position corresponding to asparagine at position 135 (N135) of the amino acid sequence set forth in SEQ ID NO: 2; and a position corresponding to threonine at position 706 (T706) of the amino acid sequence set forth in SEQ ID NO: 2.

It is preferable that the enzyme of the present invention has an amino acid residue which is different from that of natural α-glucan phosphorylase in at least two positions selected from the group consisting of: a position corresponding to phenylalanine at position 39 (F39) of the amino acid sequence of SEQ ID NO: 2; a position corresponding to asparagine at position 135 (N135) of the amino acid sequence set forth in SEQ ID NO: 2; and a position corresponding to position 705 threonine (T706) of the amino acid sequence set forth in SEQ ID NO: 2. It is most preferable that the enzyme of the present invention has an amino acid residue which is different from that of natural α-glucan

phosphorylase in all positions of a position corresponding to phenylalanine at position 39 (F39) of the amino acid sequence set forth in SEQ ID NO: 2; a position corresponding to asparagine at position 135 (N135) of the amino acid sequence set forth in SEQ ID NO: 2; and a position corresponding to position 706 threonine (T706) of the amino acid sequence offset forth in SEQ ID NO: 2.

It is thought that the aforementioned three positions of natural α-glucan phosphorylase interact with surrounding amino acids in the steric structure of α-glucan phosphorylase to form a steric partial structure which destabilizes the enzyme. By changing a residue at these positions to another amino acid residue, an enzyme is stabilized, and thermostability is improved. In addition, since residues at these positions steric-structurally interact with surrounding amino acid residues, substitution of the amino acid residues has unexpectedly important significant effects. For example, in the case of potato type L α-glucan phosphorylase, substitution of F at a position of F39 with other residues has unexpectedly important significant consequences. In addition, for example, in potato-derived type H α-glucan phosphorylase, an amino acid at a position corresponding to F39 is Y, and substitution of Y with other amino acids has unexpectedly important significant effects.

In the enzyme according to the present invention, an amino acid residue at a position corresponding to position 4 or F39 in the motif sequence 1L or 1H may be an amino acid other than an amino acid residue found in natural α-glucan phosphorylase. An amino acid residue at a position corresponding to position 4 or F39 in the motif sequence 1L or 1H is preferably an aliphatic amino acid or a heterocyclic

amino acid, more preferably an aliphatic amino acid, particularly preferably a branched amino acid(i.e. valine, leucine or isoleucine), specially preferably isoleucine or leucine, most preferably leucine.

In the enzyme according to the present invention, an amino acid residue at a position corresponding to position 4 or N135 in a motif sequence 2 can be an amino acid other than an amino acid residue found in natural α-glucan phosphorylase. An amino acid residue at a position corresponding to position 4 or N135 in a motif sequence 2 is preferably an aliphatic amino acid or a heterocyclic amino acid, more preferably alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, valine or tyrosine, particularly preferably cysteine, glycine, serine or valine.

In the enzyme according to the present invention, an amino acid residue at a position corresponding to position 7 or T706 in the motif sequence 3L or 3H can be an amino acid other than an amino acid residue found in natural α-glucan phosphorylase. An amino acid residue at a position corresponding to position 7 or T706 in a motif sequence 3L or 3H is preferably an aliphatic amino acid, more preferably a branched amino acid (i.e. valine, leucine or isoleucine) or a sulfur-containing amino acid (i.e. cysteine, cysteine, methionine), particularly preferably cysteine, isoleucine, leucine, valine or tryptophan, particularly preferably cysteine, isoleucine, leucine or valine, most preferably isoleucine.

In the method according to the present invention, for

preparing α-glucan phosphorylase having improved thermostability, a substitution, addition, deletion or modification of an amino acid can be performed in addition to alteration of the object of the invention (such the substitution that an α-glucan phosphorylase has an amino acid residue which is different from an amino acid residue of the natural α1-glucan phosphorylase in at least one position selected from the group consisting of a position corresponding to phenylalanine at position 39 (F39) of the amino acid sequence set forth in SEQ ID NO: 2, a position corresponding to asparagine at position 135 (N135) of the amino acid sequence set forth in SEQ ID NO: 2 and a position corresponding to position 706 threonine (T706) of the amino acid sequence set forth in SEQ ID NO: 2). Substitution of an amino acid refers to substitution of one amino acid with another one amino acid. Addition of an amino acid refers to insertion of one or more, for example, 1 to 10, preferably 1 to 5, more preferably 1 to 3 amino acids into any position of the original amino acid sequence. Deletion of an amino acid refers to removal of one or more, for example, 1 to 10, preferably 1 to 5, more preferably 1 to 3 amino acids from the original amino acid sequence. Examples of amino acid modification include but are not limited to amidation, carboxylation, sulfation, halogenation, alkylation, glycosylation, phosphorylation, hydroxylation, and acylation (e.g. acetylation). The α-glucan phosphorylase having improved thermostability of the present invention may be synthesized by a peptide synthesis method and, in such the case, an amino acid to be substituted or added may be a natural amino acid, a non-natural amino acid or an amino acid analog. A natural amino acid is preferable.

The α-glucan phosphorylase having improved

thermostability of the present invention may be an enzyme analog having the same enzyme activity as α-glucan phosphorylase. As used herein, a term "enzyme analog" refers to an entity which is a different compound from a natural enzyme, but has equivalent in at least one chemical function or biological function to that of a natural enzyme. Therefore, the enzyme analog includes an entity in which one or more amino acid analogs are added or substituted relative to the original natural enzyme. The enzyme analog has such an addition or substitution, that its function (e.g. α-phosphorylase activity or thermostability) is substantially the same as, or better than, the function of the original natural enzyme. Such an enzyme analog can be prepared using techniques well-known in the art. Therefore, the enzyme analog can be a polymer containing an amino acid analog. In the present specification, the "enzyme" includes this enzyme analog unless otherwise indicated.

In the present specification, the "amino acid" may be a natural amino acid, a non-natural amino acid, a derivative amino acid, or an amino acid analog. A natural amino acid is preferable.

The term "natural amino acid" means an L-isomer of a natural amino acid. A natural amino acid is glycine, alanine, valine, leucine, isoleucine, serine, methionine, threonine, phenylalanine, tyrosine, tryptophan, cysteine, proline, histidine, aspartic acid, asparagine, glutamic acid, glutamine, γ-carboxyglutamic acid, arginine, ornithine, and lysine. Unless otherwise is indicated, all amino acids referred in the present specification are in L form, and an embodiment using an amino acid in D form is also within the scope of the present invention.

The term "non-natural amino acid" means an amino acid which is not usually found in a protein in nature. Examples of the non-natural amino acid include norleucine, para-nitrophenylalanine, homophenylalanine, para-fluorophenylalanine, 3-amino-2-benzylpropionic acid, a D form or an N form of homoarginine, and D-phenylalanine.

The term "derivative amino acid" refers to an amino acid which is obtained by derivatizing an amino acid.

The term "amino acid analog" refers to a molecule which is not an amino acid, but is similar to an amino acid in physical properties and/or function. Examples of the amino acid analog include, for example, ethionine, canavanine, and 2-methylglutamine.

In the present specification, an amino acid can be referred by any of the generally known three letter symbol, and one letter symbol recommended by IUPAC-IUB Biochemical Nomenclature Commission. A nucleotide can be referred by a generally-accepted one letter code, similarly.

α-glucan phosphorylase having improved thermostability including modification due to substitution, addition or deletion of one or a few or more plural amino acids relative to an amino acid sequence of natural α-glucan phosphorylase, in addition to the objective modification is within the scope of the present invention. Such an α-glucan phosphorylase having improved thermostability including substitution, addition or deletion of one or a few or more amino acid can be prepared according to the methods described in, for example, Molecular Cloning, A Laboratory Manual, Second Edition, Cold

Spring Harbor Laboratory Press (1989), Current Protocols in Molecular Biology, Supplement 1-38, John Wiley & Sons (1987-1997), Nucleic Acids Research, 10, 6487 (1982), Proc. Natl. Acad. Sci., USA, 79, 6409 (1982), Gene, 34, 315 (1985), Nucleic Acids Research 13, 443 (1985), Proc. Natl. Acad. Sci USA, 82, 488 (1985), Proc. Natl. Acad. Sci., USA, 81, 5662 (1984), Science, 224, 1431 (1984), PCT WO 85/00817 (1985), Nature, 316, 601 (1985).

A α-glucan phosphorylase having improved thermostability according to the present invention can be prepared by utilizing methods well-known in the art. For example, deletion, substitution or addition of an amino acid in the α-glucan phosphorylase having improved thermostability of the present invention can be performed by site-directed mutagenesis which is a well-known technique. The procedure of site-directed mutagenesis is well-known in the art. For example, see Nucl. Acid Research, Vol.10, pp.6487-6500 (1982).

In the present specification, the "substitution, addition or deletion of one or a few or more plural amino acids" or the "substitution, addition or deletion of at least one amino acid", when used regarding α-glucan phosphorylase having improved thermostability, refers to a number of substitutions, additions or deletions, to such a degree that the enzyme activity of α-glucan phosphorylase is not lost, preferably, the enzyme activity becomes equivalent or superior over a standard (e.g. natural α-glucan phosphorylase). Those skilled in the art can easily select α-glucan phosphorylase having improved thermostability having the desired nature. Alternatively, objective α-glucan phosphorylase having improved thermostability may

be directly chemically synthesized. Such chemical synthesis method is well-known in the art.

The thus prepared α-glucan phosphorylase having improved thermostability of the present invention has preferably about 40%, more preferably about 45%, more preferably about 50%, more preferably about 55%, more preferably about 60%, more preferably about 65%, more preferably about 70%, more preferably about 75%, more preferably about 80%, more preferably about 85%, more preferably about 90%, more preferably about 95%, and most preferably about 99% identity to an amino acid sequence of first (natural) α-glucan phosphorylase (preferably, potato type L α-glucan phosphorylase).

Upon design of the aforementioned alteration, the hydrophobicity index of an amino acid can be considered. Significance of a hydrophobic amino acid index upon impartation interacting biological function to a protein is generally recognized in the art (Kyte. J and Doolittle, R. F. J. Mol. Biol.157 (1): 105-132, 1982). The hydrophobic nature of an amino acid contributes to the secondary structure of a produced protein and, then, defines interaction between the protein and other molecule (e.g. enzyme, substrate, receptor, DNA, antibody, antigen and the like). An amino acid is assigned a hydrophobicity index based on hydrophobicity and a nature of a charge thereof. They are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamic acid (-3.5); glutamine (-3.5); aspartic acid (-3.5); asparagine (-3.5); lysine

(-3.9); and arginine (-4.5).

It is well-known in the art to substitute a certain amino acid with another amino acid having a similar hydrophobicity index, thereby, a protein still having similar biological functions (e.g. protein equivalent in enzyme activity) can be produced. In such an amino acid substitution, a hydrophobicity index is preferably within ±2, more preferably within ±1, further preferably within ±0.5. It is understood in the art that such the substitution of an amino acid based on hydrophobicity is efficient. As described in USP No.4,554,101, the following hydrophilicity index is assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartic acid (+3.0±1); glutamic acid (+3.0 ±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5±1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). It is understood that an amino acid can be substituted with another amino acid which has a similar hydrophilicity index, and can still impart a biological equivalent. In such the amino acid substitution, the hydrophilicity index is preferably within ±2, more preferably within ±1, and further preferably within ±0.5.

In the present invention, "conservative substitution" refers to substitution in which a hydrophilicity index or/and a hydrophobicity index are similar ,as described above, between the original amino acid and an amino acid to be substituted, in amino acid substitution. Examples of conservative substitution are well-known to those skilled in the art, and include, but are not limited to substitution

among the following each group, for example: arginine and lysine; glutamic acid and aspartic acid; serine and threonine; glutamine and asparagines; and valine, leucine, and isoleucine.

(3.2 Method of assessing thermostability)

The α-glucan phosphorylase having improved thermostability of the present invention has one characteristic, in that enzyme activity of α-glucan phosphorylase having improved thermostability at 37°C, after it is heated in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is 20% or more of enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, before heating. Enzyme activity of the α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes, is preferably about 20% or more, more preferably about 25% or more, more preferably about 30% or more, more preferably about 40% or more, more preferably about 50% or more, more preferably about 55% or more, more preferably about 60% or more, further preferably about 65% or more, further preferably about 70% or more, particularly preferably about 80% or more, most preferably about 90% or more of enzyme activity of α-glucan phosphorylase having improved thermostability at 37°C , before the heating.

Enzyme activity of α-glucan phosphorylase having improved thermostability at 37°C, after heating in a 20 mM citrate buffer (pH 6.7) at 65°C for 2 minutes is preferably about 40% or more, more preferably about 45% or more, further preferably about 50% or more, further preferably about 55% or more, particularly preferably about 60% or more, most

preferably about 65% or more of enzyme activity at 37°C of α-glucan phosphorylase having improved thermostability, before the heating.

(3.2.1 Method of measuring α-glucan phosphorylase (GP) activity)

This GP enzyme activity measuring method quantitates free inorganic phosphoric acid (Pi) produced from G-1-P.

(i) 200 μl of a reaction solution (containing 12.5 mM G-1-P, 1% dextrin and an enzyme solution in a 100 mM acetate buffer (pH 6.0)) is incubated at 37°C for 15 minutes.

(ii) 800 μl of a molybdenum regent (15 mM ammonium molybdate, 100 mM zinc acetate) is added, and this is stirred to stop the reaction.

(iii) 200 μl of 568 mM ascorbic acid (pH 5.8) is added, followed by mixing.

(iv) After incubation at 37°C for 15 minutes, an absorbance at 850nm is measured using a spectrophotometer.

(v) An absorbance is measured similarly using inorganic phosphoric acid having the known concentration, and a standard curve is produced.

(vi) An absorbance value obtained for a sample is fitted to this standard curve, and the amount of inorganic phosphoric acid in the sample is determined. Inorganic phosphoric acid is quantitated as a phosphoric acid ion. The amount of glucose-1-phosphate is not quantitated. In the present

specification, one unit of α-glucan phosphorylase activity is defined an activity which produces 1 μmol inorganic phosphoric acid (Pi) for one minute as one unit (U) when measured by this measuring method .

(3.2.2 Method of measuring thermostability)

Thermostability is measured according to the following procedure.

(i) 0.2 U/ml of an enzyme solution (in 20 mM citrate buffer (pH 6.7)) is incubated at 55°C, 60°C, or 65°C for 0 to 60 minutes.

(ii) a sample of enzyme solution is taken a number of time points, and retained on ice.

(iii) The enzyme solution samples of (ii) are diluted 10-fold, and enzyme activity is measured according to a GP activity measuring method. A ratio of enzyme activity A_{after} of α-glucan phosphorylase having improved thermostability at 37°C , after heating in a 20 mM citrate buffer (pH 6.7) at 60°C for 10 minutes is calculated from enzyme activity A_{before} at 37°C of the α-glucan phosphorylase having improved thermostability before heating, by $(A_{after}) / (A_{before}) \times 100(\%)$. A ratio of enzyme activity A_{after} of α-glucan phosphorylase having improved thermostability after heating relative to enzyme activity A_{before} of the α-glucan phosphorylase having improved thermostability before heating is also referred to as remaining activity.

(3.3 Method of assessing ability to synthesize amylose)

The α-glucan phosphorylase having improved thermostability of the present invention has one characteristic in that it has the ability to synthesize a glucan (particularly, amylose) having a weight average molecular weight of preferably about 60 kDa or more, more preferably about 100 kDa or more, further preferably about 150 kDa or more, further preferably about 200 kDa or more, further preferably about 250 kDa or more, further preferably about 300 kDa or more, further preferably about 350 kDa or more, further preferably about 400 kDa or more, further preferably about 450 kDa or more, further preferably about 500 kDa or more, further preferably about 550 kDa or more, further preferably about 600 kDa or more, most preferably about 650 kDa or more. A glucan having a weight average molecular weight of about 5 kDa to about 599 kDa is hardly soluble in water, while a glucan having a weight average molecular weight of about 600 kDa or more has the particular advantage that it is water-soluble. A weight average molecular weight of a glucan synthesized by the α-glucan phosphorylase having improved thermostability of the present invention does not have a particular upper limit, but a glucan up to 1000 kDa, up to 10000kDa, up to 100000 kDa can be synthesized with excellent productivity.

The "has ability to synthesize amylose having a weight average molecular weight of 60 kDa or more" refers to when a weight average molecular weight of amylose synthesized by incubation at 37°C for 18 hours using 40 μM maltotetraose, 250 mM glucose-1-phosphate, a 200 mM acetate buffer (pH 5.5), and 4U/ml reaction solution of α-glucan phosphorylase having improved thermostability (purified enzyme), is 60 kDa or more. The ability to synthesize amylose having other weight average molecular weight is similarly defined and, for

example, "has ability to synthesize amylose having a weight average molecular weight of 600 kDa or more" refers to when a weight average molecular weight of amylose synthesized under this condition is 600 kDa or more.

A weight average molecular weigh of amylose can be measured, for example, by the following method.

Firstly, synthesized amylose is completely dissolved in 1N sodium hydroxide, this is neutralized with a suitable amount of hydrochloric acid, and about 30 to 300 μg of an aliquot of amylose is subjected to gel filtration chromatography using both a differential refractometer and a multiangular light scatter detector, thereby, obtaining an average molecular weight.

More particularly, as a column, Shodex SB806M-HQ (manufactured by SHOWA DENKO K.K.) is used and, as a detector, a multiangular light scatter detector (DAWN-DSP, manufactured by Wyatt Technology) and a differential refractometer (Shodex RI-71, manufactured by SHOWA DENKO K.K.) are used by connecting them in that order. A column is retained at 40°C, and a 0.1M sodium nitrate solution is used as an eluent at a flow rate of 1mL/min. The resulting signal is collected using a data analysis software (trade name ASTRA, manufactured by Wyatt Technology), and is analyzed using the same software, thereby, a weight average molecular weight is obtained.

(3.4 Method of assessing storage stability)

The α-glucan phosphorylase having improved thermostability according to the present invention is

preferably improved in storage stability as compared with natural α-glucan phosphorylase. In the present specification, the "improved in storage stability" refers to when the enzyme is hardly degraded during storage as compared with natural α-glucan phosphorylase.

In one embodiment, storage stability refers to stability when stored at 4°C. In this case, when the α-glucan phosphorylase having improved thermostability according to the present invention is stored at 4°C for a certain period of time after purification, the molecular weight of the enzyme protein is almost equivalent to that immediately after purification. Generally, when natural α-glucan phosphorylase is stored at 4°C for a long term, it is degraded, and the molecular weight of an enzyme protein is reduced as compared with immediately after purification. After the α-glucan phosphorylase having improved thermostability of the present invention is stored preferably at 4°C for 1 month, stored more preferably at 4°C for 3 months, stored most preferably at 4°C for 5 months, it has a molecular weight approximately equivalent to that immediately after purification.

In another aspect, storage stability refers to stability when stored at 37°C. In this case, when the α-glucan phosphorylase having improved thermostability of the present invention is stored at 37°C for a certain period of time after purification, the molecular weight of the enzyme protein is approximately equivalent to that immediately after purification. Generally, when natural α-glucan phosphorylase is stored at 37°C for a long term, it is degraded, and the molecular weight of an enzyme protein is reduced as compared with immediately after purification. In another

aspect, after the α-glucan phosphorylase having improved thermostability of the present invention is preferably stored at 37°C for 4 days, more preferably stored at 37°C for 7 days, most preferably stored at 37°C for 10 days, it has a molecular weight approximately equivalent to that immediately after purification.

Of course, the α-glucan phosphorylase having improved thermostability of the present invention can be stored at any temperature which is normally used for storage. A temperature used for storage may be any temperature between about 4°C to about 37°C (e.g. about 4°C, about 5 °C, about 10 °C, about 20 °C, about 25 °C, about 37°C and the like).

Storage stability can be assessed by any method known in the art. For example, an enzyme protein immediately after purification, and an enzyme protein which has been stored at a predetermined temperature for a certain term are subjected to polyacrylamide gel electrophoresis (Native-PAGE), and storage stability can be assessed by comparing molecular weights of these enzyme proteins.

(4. Method for producing glucan using enzyme of the present invention)

The α-glucan phosphorylase having improved thermostability of the present invention can be advantageously used in a method of synthesizing a glucan. A method of synthesizing a glucan using the α-glucan phosphorylase having improved thermostability of the present invention can be any method of synthesizing a glucan known in the art, but it is preferable to use the present α-glucan phosphorylase in a method (also referred to as SP-GP method)

of reacting sucrose phosphorylase and α-glucan phosphorylase on sucrose and a primer at the same time. The SP-GP method has an advantage that a linear glucan can be produced using an inexpensive substrate.

A method of synthesizing a glucan of the preset invention includes reacting a reaction solution containing the α-glucan phosphorylase having improved thermostability according to the present invention, a sucrose phosphorylase, sucrose, a primer, and inorganic phosphoric acid or glucose-1-phosphate, to produce a glucan.

The method of synthesizing a glucan of the present invention may be a method not based on a SP-GP method. In the case of such a method, the method of synthesizing a glucan of the present invention includes reacting a reaction solution containing the α-glucan phosphorylase having improved thermostability of the present invention, a primer, and glucose-1-phosphate, to produce a glucan.

In the present specification, the "glucan" refers to a saccharide containing D-glucose as a constituent unit, and having at least two saccharide units or more of a saccharide unit linked with an α-1,4-glucoside bond. A glucan can be a linear, branched or cyclic molecule. A linear glucan has the same meaning as that of α-1,4-glucan. In a linear glucan, saccharide units are linked only with an α-1,4-glucoside bond. A glucan containing one or more α-1,6-glucoside bonds is a branched glucan. A glucan preferably contains a linear section to some extent. A linear glucan having no branching is more preferable.

It is preferably that a glucan has a small number (i.e.

the number of α-1,6-glucoside bonds) of branches in some cases. In such the case, the number of branches is representatively 0 to 10000, preferably 0 to 1000, more preferably 0 to 500, further preferably 0 to 100, further preferably 0 to 50, further preferably 0 to 25, further preferably 0.

In the glucan of the present invention, the ratio of the number of α-1,4-glucoside bonds relative to the number of α-1,6-glucoside bonds letting α-1,6-glucoside bond to be 1, is preferably 1 to 10000, more preferably 2 to 5000, further preferably 5 to 1000, further preferably 10 to 500.

α-1,6-glucoside bond may be distributed in a glucan randomly, or may be distributed uniformly. A distribution to such an extent that a linear part of 5 or more of saccharide units is formed in a glucan is preferable.

A glucan may be constructed only of D-glucose, or may be a derivative modified to such an extent that the nature of such a glucan is not deteriorated. It is preferable that the glucan is not modified.

A glucan has a molecular weight of representatively about 8×10^3 or more, preferably about 1×10^4 or more, more preferably about 5×10^4 or more, further preferably about 1×10^5 or more, further preferably about 6×10^5 or more. A glucan has a molecular weight of representatively about 1×10^8 or less, preferably about 3×10^7 or less, more preferably about 1×10^7 or less, further preferably about 5×10^6 or less, further preferably about 1×10^6 or less. In the present invention, the molecular weight of a glucan refers to a weight average molecular weight unless otherwise described.

Those skilled in the art easily understands that a glucan having a desired molecular weight is obtained by appropriately selecting an amount of a substrate, an amount of an enzyme, a reaction time and the like used in the production method of the present invention.

The SP-GP method having excellent productivity is described in International Publication WO 02/097107 pamphlet.

In the production method of the present invention, for example, α-glucan phosphorylase having improved thermostability, sucrose phosphorylase, sucrose, a primer, inorganic phosphoric acid or glucose-1-phosphate, a buffer, and a solvent dissolving it are used as main materials. Usually, these materials are all added at reaction initiation, and any material among them may be additionally added during the reaction. In the production method of the present invention, if necessary, an enzyme selected from the group consisting of a debranching enzyme, a branching enzyme, 4-α-glucanotransferase and glycogen debranching enzyme can be used. An enzyme selected from the group consisting of: a debranching enzyme, a branching enzyme, 4-α-glucantransferase and a glycogen debranching enzyme may be added to a reaction solution from beginning of the production method of the present invention, or may be added to a reaction solution midway, depending upon the desired structure of glucan.

In the present specification, the "sucrose phosphorylase" refers to any enzyme which transfers an α-glycosyl group of sucrose to a phosphate group to perform

phosphorolysis. A reaction catalyzed by sucrose phosphorylase is represented by the following equation:

Sucrose+inorganic phosphoric acid ⇔
α-D-glucose-1-phosphate+D-fructose

Sucrose phosphorylase is contained in various organisms in a natural world. Examples of an organism producing sucrose phosphorylase include but are not limited to bacteria belonging to genus Streptococcus (e.g. Streptococcus thermophilus, Streptococcus mutans, Streptococcus pneumoniae, and Streptococcus mitis), Leuconostoc mesenteroides, Pseudomonas sp., Clostridium sp., Pullularia pullulans, Acetobacter xylinum, Agrobacterium sp., Synecococcus sp., E. coli, Listeria monocytogenes, Bifidobacterium adolescentis, Aspergillus niger, Monilia sitophila, Sclerotinea escerotiorum, and Chlamydomonas sp.

Sucrose phosphorylase can be derived from any organism producing sucrose phosphorylase. It is preferable that sucrose phosphorylase has thermostability to some extent. It is more preferable that sucrose phosphorylase, when it is present alone, has higher thermostability. For example, it is preferable that, when sucrose phosphorylase is heated at 55°C for 30 minutes in the presence of 4% of sucrose, activity which is 20% or more of activity of sucrose phosphorylase before heating, is retained. Sucrose phosphorylase can be preferably derived from a bacterium selected from the group consisting of: Streptococcus mutans, Streptococcus pneumoniae, Leuconostoc mesenteroides, Oenococcus oeni, Bifidobacterium longum, Agrobacterium vitis, Pseudomonas saccharophila, Escherichia coli and Listeria innocua, can be more preferably derived from a bacterium selected from

the group consisting of: Streptococcus mutans, Streptococcus pneumoniae, Leuconostoc mesenteroides and Oenococcus oeni, further preferably can be derived from Streptococcus mutans or Streptococcus pneumoniae.

Sucrose is a disaccharide having a molecular weight of about 342, represented by $C_{12}H_{22}O_{11}$. Sucrose is present in all plants having photosynthetic ability. Sucrose may be isolated from a plant, or may be chemically synthesized. From the viewpoint of cost, it is preferable that sucrose is isolated from a plant. Examples of a plant containing a large amount of sucrose include sugarcane, and sugar beet. Sugarcane juice contains about 20% sucrose. Sugar beet juice contains about 10 to 15% sucrose. Sucrose may be provided at any purification stage from the sap or juice of a plant containing sucrose, to purified sugar.

α-glucan phosphorylase having improved thermostability and a sucrose phosphorylase used in the production method of the present invention can be used in a reaction, respectively, even when immobilized whether it is a purified enzyme or a crude enzyme, and a reaction format may be a batch format or a continuous format. As a method of immobilization, a carrier binding method (e.g. covalent binding method, ion binding method, or physical adsorbing method), a crosslinking method or an inclusion method (lattice type or microcapsule type) can be used.

Examples of a primer include maltooligosaccharide, amylose, amylopectin, glycogen, dextrin, pullulan, coupling sugar, starch, and a derivative thereof.

In the present specification, inorganic phosphoric acid

refers to a substance which can donate a phosphate substrate in the reaction of SP. In the present specification, a phosphate substrate refers to a substance which is a raw material for the phosphate moiety of glucose-1-phosphate. It is thought that, in sucrose phosphorolysis which is catalyzed by sucrose phosphorylase, inorganic phosphoric acid acts as a substrate in a form of a phosphate ion. Since this substrate is conventionally called inorganic phosphoric acid in the art, this substrate is called inorganic phosphoric acid also in the preset specification. Inorganic phosphoric acid includes phosphoric acid and an inorganic salt of phosphoric acid. Usually, inorganic phosphoric acid is used in water containing a cation such as an alkali metal ion. In this case, since phosphoric acid, a phosphate salt and a phosphate ion are in an equilibrium state, it is not possible to discriminate between phosphoric acid and a phosphate salt. Therefore, for convenience, phosphoric acid and a phosphate salt are collectively called inorganic phosphoric acid. In the present invention, inorganic phosphoric acid is preferably any metal salt of phosphoric acid, more preferably an alkali metal salt of phosphoric acid. Preferable specific examples of inorganic phosphoric acid include sodium dihydrogen phosphate, disodium hydrogen phosphate, trisodium phosphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, tripotassium phosphate, phosphoric acid (H_3PO_4), ammonium dihydrogen phosphate, and diammonium hydrogen phosphate.

Only one kind of, or a plurality of kinds of inorganic phosphoric acids may be contained in a SP-GP reaction system at reaction initiation.

Inorganic phosphoric acid can be provided, for example,

by degrading a phosphoric acid condensate such as polyphosphoric acid (e.g. pyrophosphoric acid, triphosphoric acid and tetraphosphoric acid) or a salt thereof, by a physical, chemical or enzymatic reaction, and adding this to a reaction solution.

In the present specification, glucose-1-phosphate refers to glucose-1-phosphate ($C_6H_{13}O_9P$) and a salt thereof. Glucose-1-phosphate is preferably any metal salt of glucose-1-phosphate ($C_6H_{13}O_9P$) in a narrow sense, more preferably any alkali metal salt of glucose-1-phosphate ($C_6H_{13}O_9P$). Preferable specific examples of glucose-1-phosphate include disodium glucose-1-phosphate, dipotassium glucose-1-phosphate, and glucose-1-phosphate ($C_6H_{13}O_9P$). In the present specification, glucose-1-phosphate whose chemical formula is not drawn in a parenthesis indicates glucose-1-phosphate in a wide sense, that is, glucose-1-phosphate ($C_6H_{13}O_9P$) in a narrow sense and a salt thereof.

Only one kind of, or a plurality of kinds of glucose-1-phosphates may be contained in a SP-GP reaction system, at reaction initiation.

In the method for producing a glucan according to the present invention, when a branch is generated in the product, such as when a starting material containing α-1,6-glucoside bond is used, a debranching enzyme can be used, if necessary.

A debranching enzyme which can be used in the present invention is an enzyme which can cut an α-1,6-glucoside bond. A debranching enzyme is classified into two of isoamylase (EC 3.2.1.68) which acts well on both of amylopectin and

glycogen, and α-dextrin endo-1,6-α-glucosidase (also referred to as pullulanase) (EC3.2.1.41) which acts on amylopectin, glycogen and pullulan.

A debranching enzyme is present in microorganisms, bacteria, and plants. Examples of a microorganism producing a debranching enzyme include Saccharomyces cerevisiae, and Chlamydomonas sp. Examples of a bacterium producing a debranching enzyme include Bacillus brevis, Bacillus acidopullulyticus, Bacillus macerans, Bacillus stearothermophilus, Bacillus circulans, Thermus aquaticus, Klebsiella pneumoniae, Thermoactinomyces thalpophilus, Thermoanaerobacter ethanolicus, and Pseudomonas amyloderamosa. Examples of a plant producing a debranching enzyme include potato, sweet potato, corn, rice, wheat, barley, oat, and sugar beet. An organism producing a debranching enzyme is not limited to the above examples.

In the method according to the invention, when it is desired to generate a branch in the product, a branching enzyme can be used, if necessary.

A branching enzyme which can be used in the present invention is an enzyme which can transfer a part of an α-1,4-glucan chain to position 6 of a certain glucose residue in this α-1,4-glucan chain to make a branch. A branching enzyme is also called a 1,4-α-glucan branching enzyme, a branch making enzyme or a Q enzyme.

A branching enzyme is present in a microorganism, an animal, and a plant. Examples of a microorganism producing a branching enzyme include Bacillus stearothermophilus, Bacillus subtilis, Bacillus caldolyticus, Bacillus

lichecniformis, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus caldovelox, Bacillus thermocatenulatus, Bacillus smithii, Bacillus megaterium, Bacillus brevis, Alkalophillic Bacillus sp., Streptomyces coelicolor, Aquifex aeolicus, Synechosystis sp., E. coli, Agrobacterium tumefaceins, Thermus aquaticus, Rhodothermus obamensis, Neurospora crassa, and yeast. Examples of an animal producing a branching enzyme include mammals such as human, rabbit, rat, and pig. Examples of plants producing a branching enzyme include algae; tuber and root crops such as potatoes, sweet potato, yam, and cassava; vegetables such as spinach; cereals such as corn, rice, wheat, barley, rye, and foxtail millet; and beans such as peas, soybeans, adzuki beans, and mottled kidney beans. An organism producing a branching enzyme is not limited to the above examples.

In the method according to the invention, when a cyclic structure is generated in the product, 4-α-glucanotransferase can be used, if necessary.

4-α-glucanotransferase which can be used in the present invention is also called a disproportionating enzyme, a D-enzyme, or amylomaltase, and is an enzyme which can catalyze a sugar transferring reaction (disproportionating reaction) of maltooligosaccaride. 4-α-glucanotransferase is an enzyme which transfers a glucosyl group, or a maltosyl or maltooligosyl unit from a non-reducing terminal of a donor molecule to a non-reducing terminal of an acceptor molecule. Therefore, an enzyme reaction leads to disproportion of a polymerization degree of maltooligosaccharide which was first given. When a donor molecule and an acceptor molecule are the same, an intramolecular transfer is caused and, as a result, a product having a cyclic structure is obtained.

4-α-glucanotransferase is present in microorganisms and plants. Examples of a microorganism producing 4-α-glucanotransferase include Aquifex aeolicus, Streptococcus pneumoniae, Clostridium butylicum, Deinococcus radiodurans, Haemophilus influenzae, Mycobacterium tuberculosis, Thermococcus litralis, Thermotoga maritima, Thermotoga neapolitana, Chlamydia psittaci, Pyrococcus sp., Dictyoglomus thermophilum, Borrelia burgdorferi, Synechosystis sp., E. coli, and Thermus aquaticus. Examples of plants producing 4-α-glucanotransferase include tuber and root crops such as potatoes, sweet potatoes, yam, and cassava; cereals such as corn, rice, and wheat; and beans such as peas, and soybeans. An organism producing 4-α-glucanotransferase is not limited to the above examples.

In the method of the present invention, when a cyclic structure is generated in a product, a glycogen debranching enzyme can be used, if necessary.

A glycogen debranching enzyme which can be used in the present invention is an enzyme having two kinds of activities, α-1,6-glucosidase activity and 4-α-glucanotransferase activity. Due to 4-α-glucanotransferase activity possessed by a glycogen debranching enzyme, a product having a cyclic structure is obtained.

A glycogen debranching enzyme is present in microorganisms and animals. Examples of a microorganism producing a glycogen debranching enzyme include yeast. Examples of animals producing a glycogen debranching enzyme include mammals such as human, rabbit, rat, and pig. An

organism producing a glycogen debranching enzyme is not limited to the above examples.

A solvent used in the production method of the present invention can be any solvent as far as it is a solvent which does not deteriorate the enzyme activity of sucrose phosphorylase and α-glucan phosphorylase.

As far as a reaction producing a glucan can proceed, it is not necessary that a solvent completely dissolves materials used in the production method according to the present invention. For example, when an enzyme is carried on a solid carrier, it is not necessary that an enzyme is dissolved in a solvent. Further, it is not necessary that all of reaction materials such as sucrose are dissolved, and it is enough that a part of materials, to such an extent that a reaction can proceed, is dissolved.

A representative solvent is water. A solvent may be water in a cell lysate, accompanying sucrose phosphorylase or α-glucan phosphorylase upon the preparation of sucrose phosphorylase or α-glucan phosphorylase.

Any other substance may be contained in a solution containing an α-glucan phosphorylase, a sucrose phosphorylase, sucrose, a primer, and inorganic phosphoric acid or glucose-1-phosphate, as long as interaction between the sucrose phosphorylase and sucrose, and interaction between the α-glucan phosphorylase and the primer are not hampered. Examples of such a substance include a buffer, a component of a microorganism producing α-glucan phosphorylase (e.g. bacterium, fungus), a component of a microorganism producing sucrose phosphorylase (e.g.

bacterium, fungus), salts, and a medium component.

Amounts of these materials to be used are the known, and can be appropriately selected by those skilled in the art.

In the production method according to the present invention, firstly, a reaction solution is prepared. A reaction solution can be prepared, for example, by adding an α-glucan phosphorylase, a sucrose phosphorylase, solid sucrose, a primer, and inorganic phosphoric acid or glucose-1-phosphate to a suitable solvent. Alternatively, a reaction solution may be prepared by mixing solutions each containing an α-glucan phosphorylase, a sucrose phosphorylase, sucrose, a primer, or inorganic phosphoric acid or glucose-1-phosphate. Alternatively, a reaction solution may be prepared by mixing other solid components into a solution containing some components amongst an α-glucan phosphorylase, a sucrose phosphorylase, sucrose, a primer, and inorganic phosphoric acid or glucose-1-phosphate. Any buffer may be added to this reaction solution, if necessary, for the purpose of adjusting a pH as long as it does not inhibit an enzyme reaction. To this reaction solution may be added an enzyme selected from the group consisting of: a debranching enzyme, a branching enzyme, 4-α-glucanotransferase and a glycogen debranching enzyme, if necessary.

A reaction solution is then heated, if necessary, by the methods known in the art, to react it. A reaction temperature can be any temperature as long as the effect of the invention is obtained. When a sucrose concentration in a reaction solution at reaction initiation is about 5%

to about 100%, a reaction temperature can be representatively a temperature of about 30°C to about 75 °C. It is preferable that the temperature of a solution in this reaction step is such a temperature that activity (activities) which is about 20% or more, preferably about 30% or more of activity of at least one of, preferably activities of both of sucrose phosphorylase and α-glucan phosphorylase contained in this solution before a reaction remain(s) after a predetermined reaction time. This temperature is preferably about 55°C to about 75 °C, more preferably about 60°C to about 75 °C, further preferably about 60°C to about 70 °C, particularly preferably about 60°C to about 65°C.

A reaction time can be set to be any time, in view of the reaction temperature, the molecular weight of a glucan produced by a reaction and the remaining activity of an enzyme. A reaction time is representatively about 1 hour to about 100 hours, more preferably about 1 hour to about 72 hours, further more preferably about 2 hours to about 36 hours, most preferably about 2 hours to about 24 hours.

In this manner, a solution containing a glucan is produced.

(5. Method of synthesizing glucose-1-phosphate using an enzyme according to the present invention)

α-glucan phosphorylase having improved thermostability of the present invention can also be advantageously used in a method of synthesizing glucose-1-phosphate. A method of synthesizing glucose-1-phosphate using α-glucan phosphorylase having improved thermostability according to the present invention can be any method of synthesizing

glucose-1-phosphate known in the art.

A method of synthesizing glucose-1-phosphate of the present invention includes reacting a reaction solution containing α-glucan phosphorylase having improved thermostability of the present invention, a glucan and inorganic phosphoric acid to produce glucose-1-phosphate.

The definition of a glucan and inorganic phosphoric acid used in a method of synthesizing glucose-1-phosphate according to the present invention is the same as that in the aforementioned 4.

Amounts of materials to be used in a method of synthesizing glucose-1-phosphate are known, and can be appropriately selected by those skilled in the art.

In a method of synthesizing glucose-1-phosphate according to the present invention, firstly, a reaction solution is prepared. A reaction solution can be prepared, for example, by adding α-glucan phosphorylase, a glucan and inorganic phosphoric acid to a suitable solvent. Alternatively, a reaction solution may be prepared by mixing solutions each containing α-glucan phosphorylase, a glucan or inorganic phosphoric acid. Alternatively, a reaction solution may be prepared by mixing solid other components into a solution containing some components among α-glucan phosphorylase, a glucan, and inorganic phosphoric acid. To this reaction solution may be added any buffer, if necessary, for the purpose of adjusting a pH as far as it does not inhibit an enzyme reaction. To this reaction solution may be added a debranching enzyme, if necessary.

Then, the reaction solution is heated, if necessary, by the method known in the art, to react it. A reaction temperature can be any temperature as long as the effect of the invention is obtained. A reaction temperature can be representatively about 30°C to about 75 °C. It is preferable that the temperature of a solution in this reaction step is such a temperature that activity which is about 20% or more, more preferably about 30% or more of activity of α-glucan phosphorylase contained in this solution before a reaction, remains after a predetermined reaction time. This temperature is preferably about 55°C to about 75 °C, more preferably about 65°C to about 75 °C, further preferably about 60°C to about 70 °C, particularly preferably about 60°C to about 65°C.

A reaction time can be set to be any time, in view of a reaction temperature and the remaining activity of an enzyme. A reaction time is representatively about 1 hour to about 100 hours, more preferably about 1 hour to about 72 hours, further more preferably about 2 hours to about 36 hours, most preferably about 2 hours to about 24 hours.

In this manner, a solution containing glucose-1-phosphate is produced.

(6. Other production method using an enzyme according to the present invention)

α-glucan phosphorylase having improved thermostability according to the present invention can be used in any production methods known in the art using α-glucan phosphorylase, in addition to the aforementioned production methods. Utilization of α-glucan phosphorylase having

improved thermostability according to the present invention in these production methods can be easily performed by those skilled in the art.

(7. Use of glucan obtained by production method according to the present invention)

A glucan obtained by the production method according to the present invention can be used in use known in the art regarding a glucan. Among a glucan, particularly, insoluble amylose, the same function as that of dietary fiber is predicted, and utilization in a health food can be expected. Further, since amylose has the characteristic of being capable of including, for example, iodine or fatty acids in a molecule, use in the field of medicaments, cosmetics or sanitary products is expected. Amylose can be utilized as a raw material for producing cyclodextrin and cycloamylose having the same inclusion ability as that of amylose. Further, a film containing amylose has a tensile strength comparable to that of a general-use plastic, and is very promising as a material for a biodegradable plastic. In this manner, many uses are expected in amylose.

(8. Use of glucose-1-phosphate obtained by synthesis method according to the present invention)

Glucose-1-phosphate obtained by the synthesis method according to the present invention can be used in uses known in the art regarding glucose-1-phosphate. Glucose-1-phosphate is utilized as a medical antibacterial agent, an anti-tumor agent (as a platinum complex), a drug to treat heart disease (as an amine salt), or a substrate for synthesizing a glucan.

The present invention will be explained below based on Examples, but the following Examples are provided only for the purpose of exemplification. Therefore, the scope of the present invention is not limited by the aforementioned Detailed Explanation of the Invention and the following Examples, but is limited only by claims.

Examples

(1. Measurement method and calculation method)

Respective substances in the present invention were measured suing the following measurement methods.

(1.1 Quantitation of glucose)

Glucose was quantitated using a commercially available measuring kit. Glucose is measured using a glucose AR-II color developing reagent (manufactured by Wako Pure Chemical Industries, Ltd.).

(1.2 Quantitation of fructose)

Fructose was quantitated using a commercially available measuring kit. Fructose is measured using F-kit, D-glucose/D-fructose (manufacture by Roche).

(1.3 Quantitation of glucose-1-phosphate)

Glucose-1-phosphate was quantitated by the following method. To 300 μl of a measuring reagent (200 mM Tris-HCl (pH 7.0), 3 mM NADP, 15 mM magnesium chloride, 3 mM EDTA,

15 μM glucose-1,6-diphosphate, 6 μg/ml phosphoglucomutase, 6 μg/ml glucose-6-phosphate dehydrogenase) is added 600μl of a solution containing properly diluted glucose-1-phosphate, this is stirred, and the resulting reaction mixture is reacted at 37°C for 30 minutes. Thereafter, absorbance at 340nm is measured using a spectrophotometer. Absorbance is measured similarly using sodium glucose-1-phosphate having a known concentration, to produce a standard curve. An absorbance obtained for a sample is fitted to this standard curve to obtain a glucose-1-phosphate concentration in a sample. Usually, activity of producing one μmol glucose-1-phosphate for 1 minute is defined as one unit. In this quantitation method, only glucose-1-phosphate is quantitated, and an amount of inorganic phosphoric acid is not quantitated.

(1.4 Quantitation of inorganic phosphoric acid)

Inorganic phosphoric acid was obtained as phosphate ions by the following method. Into a solution (200 μl) containing inorganic phosphoric acid is mixed 800μl of a molybdenum reagent (15 mM ammonium molybdate, 100 mM zinc acetate), subsequently, 200 μl of 568 mM ascorbic acid (pH 5.0) is added, this is stirred, and the resulting reaction mixture is reacted at 37°C for 30 minutes. Thereafter, absorbance at 850nm is measured using a spectrophotometer. Absorbance is measured similarly using inorganic phosphoric acid having the known concentration, to produce a standard curve. An absorbance obtained for a sample is fitted to this standard curve, to obtain a measure of the inorganic phosphoric acid in a sample. In this quantitation method, the amount of inorganic phosphoric acid is quantitated, and the amount of a glucose-1-phosphate is not quantitated.

(1.5 Method of calculating yield of glucan produced from glucose-1-phosphate)

A yield of a glucan (e.g. amylose) produced using α-glucan phosphorylase and, as a starting material, glucose-1-phosphate without using sucrose phosphorylase is obtained by the following equation from amounts of inorganic phosphoric acid and glucose in a solution after reaction termination.

(Glucan yield (%))
=(glucose used in glucan synthesis (mM))÷(initial glucose-1-phosphate (mM))×100
={(inorganic phosphoric acid produced by reaction (mM))-(glucose after reaction (mM))}÷(initial glucose-1-phosphate (mM))×100

(1.6 Method of calculating yield of glucan produced from sucrose)

A yield of a glucan (e.g. amylose) produced using inorganic phosphoric acid as a staring substance in a SP-GP method is obtained by the following equation from amounts of glucose, fructose, and glucose-1-phosphate in a solution after reaction termination.

Glucan (mM glucose equivalent)
=(fructose (mM)-(glucose-1-phosphate (mM))-(glucose (mM))

This equation is based on the following principle.

In the production method of the present invention,

firstly, a reaction (A) according to the following equation can occur.

(A) sucrose +inorganic phosphoric acid → glucose-1-phosphate+fructose

This reaction is catalyzed by sucrose phosphorylase. In this reaction, sucrose and inorganic phosphoric acid are reacted to produce the same molar amounts of glucose-1-phosphate and fructose. Since the resulting fructose reacts with other substance no longer, a molar amount of produced glucose-1-phosphate is known by measuring a molar amount of fructose.

Sucrose phosphorylase can catalyze hydrolysis of sucrose of the following reaction (B) as a side reaction in addition to the aforementioned reaction (A).

(B) Sucrose →glucose+fructose

An amount of glucose incorporated into a glucan is calculated as follows.

Amount of glucose incorporated into a glucan
=(amount of glucose-1-phosphate produced by reaction (A))-(amount of unreacted glucose-1-phosphate)
=(amount of fructose produced by reaction (A))-(amount of unreacted glucose-1-phosphate)

In view of the fructose produced by a reaction (B), the amount of fructose produced by a reaction (A) is calculated as follows:

Amount of fructose produced by reaction (A)
=(amount of fructose after reaction termination)-(amount of glucose after reaction termination)

Therefore, a yield of a glucan is obtained by the following equation.

(Glucan (mM glucose equivalent))
=(fructose (mM))-(glucose-1-phosphate (mM))-(glucose (mM))

A yield of a glucan produced using glucose-1-phosphate as a starting material is obtained by the following equation from an amount of initial glucose-1-phosphate, as well as amounts of glucose, fructose and glucose-1-phosphate in a solution after reaction termination.

(Glucan (mM glucose equivalent))

=(initial glucose-1-phosphate (mM))
+(fructose (mM)-(glucose (mM))
-(glucose-1-phosphate after reaction (mM))

This equation is based on the following principle.

In a reaction solution, in addition to initial glucose-1-phosphate, glucose-1-phosphate is produced by a reaction (A). That is, initial glucose-1-phosphate and produced glucose-1-phosphate can be used in glucan synthesis. By subtracting the amount of glucose-1-phosphate remaining in a reaction solution after reaction termination, from the amount of glucose-1-phosphate which can be used in glucan synthesis, the amount of glucose-1-phosphate used in a reaction, that is, an amount of glucose incorporated into

a glucan can be calculated. Therefore, an amount of glucose incorporated into a glucan can be obtained by the aforementioned equation. This equation can be also applied when inorganic phosphoric acid and glucose-1-phosphate are used together as a starting material in a SP-GP-reaction system.

(1.7 Method of calculating yield of glucan produced from sucrose)

A yield of a glucan when produced using inorganic phosphoric acid as a starting material is obtained by the following equation.

(Glucan yield (%))

=(glucan (mM glucose equivalent)) / (initial sucrose (mM)) × 100

A yield of a glucan when produced using glucose-1-phosphate as a starting material is obtained by the following equation.

(Glucan yield (%))
={(initial glucose-1-phosphate (mM))+(fructose (mM))-(glucose (mM)-(glucose-1-phosphate after reaction (mM))}÷{(initial sucrose (mM))+(initial glucose-1-phosphate (mM))}×100

This equation can be also applied when inorganic phosphoric acid and glucose-1-phosphate are used together as a starting material in a SP-GP reaction system.

(Example 1: Preparation, screening and sequencing of potato α-glucan phosphorylase having improved thermostability)

To briefly outline, a random mutation was introduced into a potato-derived type L α-glucan phosphorylase gene, a gene with a random mutation introduced therein was introduced into Escherichia coli, α-glucan phosphorylase with a random mutation introduced therein was expressed and, Escherichia coli expressing, among expressed α-glucan phosphorylases, α-glucan phosphorylase having improved thermostability having the ability to synthesize a glucan after heating at 60°C for 10 minutes was selected, a gene of α-glucan phosphorylase having improved thermostability was isolated from this Escherichia coli, and the sequence thereof was determined.

Details are as follows.

Firstly, a gene of potato-derived type L α-glucan phosphorylase (GP) was prepared. According to the description of Takaha, et. al. (Journal of Biological Chemistry, vol.268, pp.1391-1396, 1993), an mRNA was prepared from a potato tuber using a well-known method, and a cDNA library was prepared using a commercially available kit.

Then, based on the known GP gene sequence (database GenBank accession number D00520), PCR primer 1 and PCR primer 2 were designed. Employing the aforementioned cDNA library as a template, and using, as PCR primers 1 and 2,

PCR primer 1: 5'AAATCGATAGGAGGAAAACAT ATG ACC TTG AGT GAG AAA AT 3' (SEQ ID NO: 38)

and

PCR primer 2: 5'GAAGGTACCTTTTCATTCACTTCCCCCTC3' (SEQ ID NO: 39),

PCR was performed to amplify a gene of potato-derived GP. PCR conditions were a PCR reaction of 30 cycles, one cycle being 94°C for 30 second, 50°C for 1 minute, and 72 °C for 3 minutes. The underlined part of PCR primer 1 corresponds to a structural gene sequence at the N-terminal region of a type L GP mature protein, and an underlined part of PCR primer 2 corresponds to a base sequence immediately after a stop codon of a type L GP structural gene.

The amplified GP gene was inserted into a plasmid pMW118 (manufactured by Nippon Gene Co., Ltd.) which had been previously cut with SmaI, and a plasmid having a sequence such as in Fig.2 was selected. This plasmid was introduced into Escherichia coli TG-1 by a calcium phosphate precipitation method, an ampicillin resistant strain was selected, this ampicillin resistant strain was cultured, and a plasmid was recovered from this ampicillin resistant strain, thereby, the gene of a potato-derived type L GP was obtained.

The resulting gene of a potato-derived type L GP, was amplified by an error-prone PCR method known to those skilled in the art (References; Leung, et. al. (Technique 1, 11-15, 1989) and Cadwell and Joyce (PCR Methods Applic.2,28-33, 1992),using PCR primer 3 and PCR primer 4,

PCR primer 3: 5'-TTCGGATCCTCACCTTGAGTGAGAAAATTCAC-3' (SEQ ID NO: 40)

and

PCR primer 4: 5'-TTCGGATCCTTTTCATTCACTTCCCCCTC-3' (SEQ ID NO: 41),

a PCR reaction of 90 °C for 30 seconds, thereafter, 25 cycles, one cycle being 94°C for 30 second, and 68 °C for 3 minutes, thereafter, 68 °C for 1 minute was performed. Base substitution was introduced into an average 2 to 3 positions of the amplified DNA fragment. The underlined part of PCR primer 3 corresponds to a structural gene sequence at the N-terminal region of a type L GP mature protein, and the underlined part of PCR primer 4 corresponds to a base sequence immediately after a stop codon of a type L GP structural gene.

A GP gene amplified fragment with a random mutation introduced therein was inserted into a plasmid pET3d (manufactured by STRATAGENE) which had been previously cut with BamHI, and a plasmid library for screening GP having improved thermostability with a random mutation introduced therein was prepared. Escherichia coli BL21 (DE3) was transformed with this plasmid, and a transformant was diluted so that an independent colony was obtained, and plated on an ampicillin-containing LB agar medium (50 μg/ml ampicillin, tryptone 1% manufactured by Difco, yeast extract 0.5% manufactured by Difco, NaCl 0.5%, 1.5% agarose, pH 7.3), followed by culturing at 30°C for 24 hours. Colonies on the resulting plate were transferred onto a nylon membrane filter. The surface of a filter on which colonies were adhered was sufficiently dried, and this filter was incubated at 60°C for 10 minutes in a 20 mM citrate buffer (pH 6.7). After

transfer, the remaining plate was further incubated at 37°C for a few hours and, thereafter, was stored at 4°C as a master plate. The heat-treated filter was applied to a gel (containing 0.05% dextrin, 50 mM G-1-P, 100 mM citrate buffer (pH 6.7), 0.7% agarose) containing a substrate for glucan synthesis so that a colony-adhered surface was adhered to a gel surface, and this was incubated at 50°C for 2 hours. The filter peeled from the gel was immersed in an iodine solution (0.1% potassium iodide, 0.01% iodine), and glucan synthesized on the filter was detected by an iodine starch reaction. Colonies corresponding to spots stained with blue were isolated from a master plate.

From the thus obtained each Escherichia coli, a plasmid was recovered according to a method known in the art, and the base sequence of a gene of α-glucan phosphorylase having improved thermostability in this plasmid was determined using a DNA sequencer (manufactured by ABI).

When an amino acid sequence encoded by this gene of α-glucan phosphorylase having improved thermostability was compared with an amino acid sequence of natural potato type L (i.e. before mutation) α-glucan phosphorylase, a mutation was introduced into amino acids at position 39, position 135 or position 706 of natural potato type L α-glucan phosphorylase, and the amino acids were substituted as F39 → L, N135 → S, or T706 → I, respectively. In addition, improvement in thermostability was also seen in a GP in which F39 was mutated into an amino acid other than L, N135 was mutated into an amino acid other than S, or T706 was mutated into an amino acid other than I.

(Example 2-1A: Preparation of potato type L α-glucan

phosphorylase having improved thermostability by site-directed mutagenesis)

In the present Example, GP having improved thermostability having only one substitution at a position which was found to contribute to improve thermostability in Example 1, GP having improved thermostability having a combination of any two, and GP having improved thermostability having all of 3 were prepared. As an example, an amino acid sequence of GP having improved thermostability having three all mutations (F39L+N135S+T706I) is set forth in SEQ ID NO: 34, and a base sequence encoding such is set forth in SEQ ID NO: 33. For comparison, GP in which amino acids at position 39, position 135 and position 706 was not substituted, and an amino acid at a position having no relationship with these amino acid positions was substituted (GP in which only lysine at 467 position was substituted with asparagine, and GP in which only threonine at position 711 was substituted with alanine) was prepared. Many methods of substituting an amino acid are published (Reference: Kinkel, T.A., Proc.Natl.Acad. Sci. USA, 82: 488 (1995), Vandeyar, M., et al., Gene, 65:129-133 (1988), Sugimoto, M., et al., Anal. Biochem., 179:309-311 (1989), Taylor, J. W. and Eckstein, F., Nucl. Acids Res., 13:8764 (1985), Nelson, M. and McClelland, M., Methods Enzymol., 216:279-303 (1992)). In the present invention, a Quick change XL Site-Directed Mutagenesis kit (manufactured by STRATAGENE) was used. Employing a plasmid containing a potato-derived type L GP gene inserted in a plasmid pMW-118 shown in Example 1 as a template, and using one set of mutation-introduced primers per mutation, each being about 35bp complementary relative to a central, mutation-introduced position, and were designed to introduce a mutation of F39L, N135S, T706I, K467D or T711A,

PCR was performed to carry out site-directed mutagenesis. A plasmid pMW-PGP containing the thus obtained gene encoding GP having improved thermostability was prepared. Escherichia coli TG-1 was transformed with this plasmid, and a transformant was diluted so that an independent colony was obtained, and plated on an ampicillin-containing LB agar medium (50 μg/ml ampicillin, tryptone 1% manufactured by Difco, yeast extract 0.5% manufactured by Difco, NaCl 0.5%, 1.5% agarose, pH 7.3), followed by culturing at 37°C overnight. Escherichia coli grown on this ampicillin-containing LB agar medium harbors an introduced plasmid. In this manner, Escherichia coli expressing GP having improved thermostability was prepared. By extracting a plasmid from the resulting Escherichia coli, and sequencing a gene encoding GP, it was confirmed that a plasmid contained in Escherichia coli obtained in the present Example has a mutant GP gene encoding GP having improved thermostability and having an objective mutation.

It was confirmed as follows that GP expressed by Escherichia coli obtained in the present Example is has improved thermostabilty. Escherichia coli TG-1 harboring an introduced plasmid was inoculated on an ampicillin-containing LB medium (50 μg/ml ampicillin, tryptone 1% manufactured by Difco, yeast extract 0.5% manufactured by Difco, NaCl 0.5%, pH 7.3), this was grown at 37°C to a logarithmic middle phase, the temperature was lowered to around 22 °C, and ispopropyl β-D-thiogalactoside which is a gene expression inducer was added to a final concentration of 0.1 mM, and pyridoxine hydrochloride was added to a final concentration of 1 mM, followed by culturing at 22 °C for about 20 hours. The culture was centrifuged to recover bacterial cells, the bacterial cells were

suspended in a buffer, and the suspension was sonicated to obtain a bacterial cell extract. This bacterial extract was treated at 60°C for 30 minutes to obtain an authentic GP preparation.

When a glucan was produced using the resulting authentic GP preparation by a method of reacting sucrose phosphorylase and α-glucan phosphorylase on sucrose and a primer (method described in International Publication WO 02/097107 pamphlet) , a high-molecular glucan could be obtained at a high yield, with respect to all of α-glucan phosphorylase having improved thermostability.

On the other hand, GP in which an amino acid at a position having no relationship with improvement of thermostability had been substituted was inactivated by treatment at 60°C for 30 minutes, and a glucan could not be produced.

(Example 2-1B: Preparation of modified potato type L α-glucan phosphorylase substituted with various amino acids)

According to the same manner as that of Example 2-1A, except that primers designed so that one place of F39, N135 and T706 was substituted with another amino acid residue were used, a plasmid containing a modified α-glucan phosphorylase gene was prepared, and various modified GP authentic preparations were obtained.

Thermostability of these modified GP authentic preparations were studied in detail in the Example 3-1 (3-1) below.

(Example 2-2A: Preparation of potato type H α-glucan

phosphorylase having improved thermostability by site-directed mutagenesis)

According to the same manner as that of Example 2-1A except that a potato-derived type H α-glucan phosphorylase gene was used in place of a potato-derived type L α-glucan phosphorylase gene, a plasmid containing a gene of α-glucan phosphorylase having improved thermostability was prepared, and a GP authentic preparation was obtained. In the present Example, a GP having improved thermostability having only one substitution at a position corresponding to N135S or T706I of an amino acid sequence of potato type L α-glucan phosphorylase (position 133 and position 628 of an amino acid sequence of potato type H α-glucan phosphorylase, respectively) among substitution positions which were found to contribute to improvement of thermostability in Example 1, was prepared.

When these GP authentic preparations were used to perform a treatment at 60°C for 30 minutes as in Example 2-1A, and a glucan was prepared, with respect to all of the α-glucan phosphorylase having improved thermostability, a high-molecular weight glucan could be obtained, similar to natural potato type H α-glucan phosphorylase.

(Example 2-2B: Preparation of potato type H α-glucan phosphorylase having improved thermostability by site-directed mutagenesis)

According to the same manner as that of Example 2-1A, except that a potato-derived type H α-glucan phosphorylase gene was used in place of a potato-derived type L α-glucan phosphorylase gene, and a mutation-introduction primer

designed so that amino acid residues of a position corresponding to F39 (Y36), a position corresponding to N135 (N133) and a position corresponding to T706 (T628) were substituted with leucine(L), serine (S) and isoleucine (I), respectively, was used, a plasmid containing a gene of α-glucan phosphorylase having improved thermostability was prepared, and a triple mutant (Y36L+N133S+T628I) GP authentic preparation was obtained. In the present Example, a GP having improved thermostability having substitutions at all three positions which had been found to contribute to improvement of thermostability in Example 1 was prepared.

Heat resistance of these modified GP authentic preparations were studied in detail in Example 3-2 (2) below.

(Example 2-2C: Preparation of Arabidopsis thaliana type H α-glucan phosphorylase having improved thermostability by site directed mutagenesis)

Firstly, a gene of Arabidopsis thaliana-derived type H α-glucan phosphorylase (GP) was prepared using a commercially available Arabidopsis-derived cDNA (PCR Ready First Strand cDNA, manufactured by Wako Pure Chemical Industries, Ltd.).

More particularly, based on the known Arabidopsis thaliana GP gene sequence (database GenBank accession number AL133292; CAB61943.1), PCR primers 5 and 6 were designed. Employing the aforementioned Arabidopsis-derived cDNA as a template, and using:

PCR primer 5: 5'AAATCGATAGGAGGAAAACAT ATG GCA AAC GCC AAT GGA AAA GCT GCG ACT AGT TTA CCG GAG AAA ATC TC 3' (SEQ ID

NO: 42)

and

PCR primer 6: 5'GAAGGTACC TTA GGG AAC AGG ACA AGC CTC AAT GTT CCA AAT CTC TTT GGC ATA CTG AG 3' (SEQ ID NO: 43),

PCR was performed to amplify an Arabidopsis thaliana-derived type H GP gene. The conditions of the PCR reaction were 30 cycles, one cycle being 94°C for 30 seconds, 60°C for 1 minute, and 72 °C for 3 minutes. The underlined part of PCR primer 1 corresponds to a structural gene at the N-terminal region of a mature protein of an Arabidopsis thaliana-derived type H GP gene, and the underlined part of PCR primer 2 corresponds to a structural gene at the C-terminal region of a mature protein of an Arabidopsis thaliana-derived type H GP gene.

The amplified Arabidopsis thaliana-derived type H GP gene was inserted into a plasmid pMW118 (manufactured by Nippon Gene Co., Ltd.) which had been previously cut with SmaI, this plasmid was introduced into Escherichia coli TG-1 using a competent cell method, an ampicillin resistant strain was selected, this ampicillin resistant strain was cultured, and a plasmid was recovered from this ampicillin resistant strain, thereby, an Arabidopsis thaliana-derived type H GP gene was obtained.

According to the same manner as that of Example 2-1A except that the resulting Arabidopsis thaliana-derived type H GP gene was used in place of a potato-derived type L α-glucan phosphorylase gene, and a mutation-introduction promoter designed so that amino acid residues at a position corresponding to F39 (Y40), a position corresponding to N135

(N136) and a position corresponding to T706 (N631) were substituted with leucine (L), serine (S) and isoleucine (I), respectively, was used, a plasmid containing a gene of α-glucan phosphorylase having improved thermostability was prepared, and a triple mutant (Y40L+N136S+N631I) GP authentic preparation was obtained. In the present Example, a GP having improved thermostability having substitutions at all three positions which had been found to contribute to improvement of thermostability in Example 1 was prepared.

Thermostability of these modified GP authentic preparations were studied in detail in Example 3-2 (2) below.

(Example 3-1: Preparation of various α-glucan phosphorylases having improved thermostability, on a large scale, and comparison of thermostability)

(1) Large scale preparation of enzyme

Respective Escherichia coli's expressing a GP having improved thermostability prepared in Example 2-1A or 2-1B were cultured in a TB medium (containing Terrific broth (GIBCO) 47g/L, glycerol 4ml/L and 50 μg/ml ampicillin) at 37°C for 5 hours, IPTG and pyridoxine chloride were added to this culture solution to final concentrations of 0.1 mM IPTG and 1 mM pyridoxine hydrochloride, and this was further cultured at 22 °C for 24 hours. Then, bacterial cells were recovered by centrifuging the culture, medium components were removed by washing with a 20 mM citrate buffer. Bacterial cells after washing were suspended in a 20 mM citrate buffer, bacterial cells were lysed with an sonicator, and centrifuged, and the supernatant was used as a bacterial cell extract. The resulting bacterial cell extract was

loaded on a Q-Sepharose FF column which had been previously equilibrated, and a fraction containing a GP having improved thermostability eluting at a concentration gradient of 0.1M to 0.3M NaCl in a 20 mM citrate buffer (pH 6.7) was recovered. The recovered enzyme fraction was loaded on a Phenyl-TOYOPEARL 650M column which had been previously equilibrated, and a fraction containing a GP having improved thermostability-containing fraction eluting at a concentration gradient of 17.5% to 7.5% saturated ammonium sulfate in a 20 mM citrate buffer was recovered. The recovered enzyme fraction was loaded on a HiTrap HQP column which had been previously equilibrated, and an active fraction eluting at a concentration gradient of 0.1M to 0.4M NaCl in a 20 mM citrate buffer was recovered. The resulting active fragment was further loaded on a Resource Q column which had been previously equilibrated, and this was eluted at a concentration gradient of 0.1M to 0.4M NaCl in a 20 mM citrate buffer, to recover a purified enzyme-containing active fragment.

The resulting purified enzyme-containing active fragment was subjected to about 1 μg native PAGE (Native polyacrylamide gel electrophoresis). As a result, for all Escherichia coli expressing GP having improved thermostability, a single band was recognized at a molecular weight of about 210 kDa, and no band was seen at any other place. Since GP is predicted to have a molecular weight of about 104 kDa from an amino acid sequence, it is thought that GP takes a dimer structure. In this manner, it was shown that GP having improved thermostability was uniformly purified.

(2) Measurement of activity of purified GP having improved

thermostability

Activity of GP having improved thermostability purified in the (1) was measured. Measurement was performed as follows. Firstly, a 200 µl reaction solution (containing 12.5 mM G-1-P, 1% dextrin and an enzyme solution in a 100 mM acetate buffer (pH 6.0)) was incubated at 37°C for 15 minutes. Then, 800 µl of a molybdenum reagent (15 mM ammonium molybdate, 100 mM zinc acetate) was added, and stirred to stop the reaction. Then, 200 µl of 568 mM ascorbic acid (pH 5.8) was added, this was mixed, and incubated at 37°C for 15 minutes, and an absorbance at 850nm was measured using a spectrophotometer. In the present Example, GP enzyme activity was measured by quantitating free inorganic phosphoric acid produced from G-1-P. An amount of an enzyme producing 1 µmol inorganic phosphoric acid for one minute was defined as one unit (U).

(3-1) Comparison of thermostability at 60°C and 65°C of GP having improved thermostability prepared in Example 2-1A

Thermostability at 60°C and 65°C of respective GPs having improved thermostability which had been prepared in Example 2-1A, and prepared at a large scale and purified in the (1) were compared. As a control, natural (not mutated) potato type L α-glucan phosphorylase purified by the same method was used.

Firstly, 0.2U/ml of a purified enzyme solution (in a 20 mM citrate buffer (pH 6.7)) was incubated at 60°C or 65°C for 0 to 30 minutes. An aliquot of an enzyme solution was taken out at specific time points, such as 0, 2, 10, 20 and 30 minutes, and retained on ice. Samples of enzyme solution retained on ice was 10-fold diluted with a 20 mM citrate

buffer (pH 6.7), and enzyme activity was measured according to the activity measuring method described in (2). Thermostability of an enzyme was judged by a ratio of enzyme activity at 37°C of an enzyme after incubation (i.e. remaining activity), when the enzyme activity at 37°C of an enzyme before incubation at 60°C or 65°C is taken to be 100%. Results of incubation at 60°C are shown in the following Table 5. Results of incubation at 65°C are shown in the following Table 6.

Table 5

Remaining activity (%) when incubated at 60°C

Time (min)	Natural potato type L	F39L	N135S	T706I	F39L +N135S	F39L +T706I	N135S +T706I	F39L +N135S +T706I
0	100	100	100	100	100	100	100	100
10	8.4	61.2	65.4	70.5	101	100	101	98.8
20	1.2	58.3	55.2	50.8	99.6	100	100	96.3
30	0.7	34.7	52.1	36.6	98.3	101	98.5	94.6

Table 6

Remaining activity (%) when incubated at 65°C

Time (min)	Natural potato type L	F39L	N135S	T706I	F39L +N135S	F39L +T706I	N135S +T706I	F39L +N135S +T706I
0	100	100	100	100	100	100	100	100
2	1.3	40.2	86.5	22.9	86.8	50.8	61.9	90.3
10	0	0.5	1.4	0.3	18.2	9.3	16.9	61.1
20	0	0.4	0.6	0.3	2.9	0.9	2.8	47.7
30	0	0.4	0.2	0.3	0.2	0.2	0.7	31.4

In the above Table 5 and Table 6, natural potato type L indicates natural potato-derived type L α-glucan phosphorylase. F39L indicates natural potato-derived type L α-glucan phosphorylase in which phenylalanine at position 39 is substituted with leucine. T706I indicates natural potato-derived type L α-glucan phosphorylase in which threonine at position 706 is substituted with isoleucine. N135S indicates natural potato-derived type L α-glucan

phosphorylase in which asparagine at position 135 is substituted with serine. F39L+T706I indicate natural potato-derived type L α-glucan phosphorylase in which phenylalanine at position 39 is substituted with leucine, and threonine at position 706 is substituted with isoleucine. N135S+T706I indicates natural potato-derived type L α-glucan phosphorylase in which asparagine at position 135 is substituted with serine, and threonine at position 706 is substituted with isoleucine. F39L+N135S indicates natural potato-derived type L α-glucan phosphorylase in which phenylalanine at position 39 is substituted with leucine, and asparagine at position 135 is substituted with serine. F39L+N135S+T706I indicates natural potato-derived type L α-glucan phosphorylase in which phenylalanine at position 39 is substituted with leucine, asparagine at position 135 is substituted with serine, and threonine at position 706 is substituted with isoleucine. Among the results presented in Table 5 and Table 6, the results of heating at 60°C for 30 minutes and results of heating at 65°C for 2 minutes are shown in Fig. 3 as a graph.

It was found that GP having improved thermostability of the present invention has very improved thermostability as compared with natural potato type L GP. From GP inferior in thermostability to GP excellent in thermostability is put in order as follows: natural potato type L GP < F39L < T706I < N135S< F39L+T706I < N135S+T706I < F39L+N135S < F39L+N135S+T706I. By substitution at only one place among amino acid residues at three places contributing thermostability improved thermostability. Further, it was seen that, by multiple substitution of these amino acid residues, thermostability is dramatically improved.

(3-2) Comparison of thermostabilities at 60°C and 65°C of modified GP prepared in Example 2-1B

Heat resistances at 60°C and 65°C of respective modified GPs which had been prepared in Example 2-1B, and prepared on a large scale and purified in Example 3-1 (1) were compared. As a control, natural (not mutated) potato-derived type L α-glucan phosphorylase purified by the same method was used.

Firstly, 0.2U/ml of a purified enzyme solution (in a 20 mM citrate buffer (pH 6.7)) was incubated at 60°C for 10 minutes or 65°C for 2 minutes. At a predetermined time (10 minutes or 2 minutes), an aliquot of an enzyme solution was taken out, and retained on ice. Samples of enzyme solution retained on ice were 10-fold diluted with a 20 mM citrate buffer (pH 6.7), and enzyme activity was measured according to the activity measuring method described in the (2). Thermostability of an enzyme was judged by a ratio of enzyme activity at 37°C of an enzyme after incubation (i.e. remaining activity), when enzyme activity at 37°C of the enzyme before incubation at 60°C for 10 minutes or 65°C for 2 minutes is taken to be 100%. Results are shown in the following Table 7 and Figs. 8 to 10.

Table 7

Remaining activity of position 39 substituted mutant

	Remaining activity (%)	
F39	60℃ 10 min	65℃ 2 min
WT	8.4	1.3
I	45.2	14.5
L	61.2	40.2
V	21.6	3.3

Remaining activity of position 706 substituted mutant

	Remaining activity (%)	
T706	60℃ 10 min	65℃ 2 min
WT	8.4	1.3
C	65.4	31.6
I	70.5	22.9
L	57.6	57.8
V	68.7	59.2
W	24.4	2.9

Remaining activity of position 135 substituted mutant

	Remaining activity (%)	
N135	60℃ 10 min	65℃ 2 min
WT	8.4	1.3
A	76.2	79.0
C	85.0	76.9
D	42.8	26.7
E	20.3	24.0
G	85.2	58.4
H	48.4	19.6
I	60.0	26.0
L	27.8	15.6
M	59.4	52.6
F	43.5	35.5
S	65.4	86.5
T	73.4	62.4
V	82.8	79.3
Y	44.8	35.4

In the above Table 7, WT indicates natural potato-derived type L α-glucan phosphorylase. In each row, an amino acid represented by one letter abbreviation indicates an amino acid substituted in a modified GP. For example, an entity expressed by I in a row labeled with F39 at a left end indicates modified GP in which phenylalanine (F) at position 39 is substituted with isoleucine (I). This is also true for modified GP in other rows.

One letter abbreviation of an amino acid is well-known to those skilled in the art, and is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

As a result, it was seen that, when amino acids at position 39, position 135 and position 706 are substituted with amino acids other than particular amino acids substituted in above Example 2-1A, the thermostability of natural potato-derived type L GP is improved.

Seeing remaining activity after incubation at 60°C for 10 minutes, when phenylalanine at position 39 was substituted with isoleucine, leucine or valine, thermostability of modified GP was superior over that of natural potato-derived type L GP. Regarding substitution at position 39, substitution with leucine (remaining activity after incubation at 60°C for 10 minutes is 61.2%) was most excellent with respect to thermostability. When asparagine at position 135 was substituted with alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine,

methionine, phenylalanine, serine, threonine, valine or tyrosine, thermostability of modified GP was superior over that of natural potato-derived type L GP. Regarding substitution at position 135, substitution with alanine (remaining activity after incubation at 60°C for 10 minutes is 76.2%), cysteine (remaining activity after incubation at 60°C for 10 minutes is 85.0%), glycine (remaining activity after incubation at 60°C for 10 minutes is 85.2%), isoleucine (remaining activity after incubation at 60°C for 10 minutes is 60.0%), serine (remaining activity after incubation at 60°C for 10 minutes is 65.4%), threonine (remaining activity after incubation at 60°C for 10 minutes is 73.4%) or valine (remaining activity after incubation at 60°C for 10 minutes is 82.8%) was particularly excellent with respect to thermostability. When threonine at position 706 was substituted with cysteine, isoleucine, leucine, valine or tryptophan, thermostability of modified GP was superior to that of natural potato-derived type L GP. Regarding substitution at a position 706, substitution with cysteine (remaining activity after incubation at 60°C for 10 minutes is 65.4%), isoleucine (remaining activity after incubation at 60°C for 10 minutes is 70.5%) or valine (remaining activity after incubation at 60°C for 10 minutes is 68.7%) was particularly excellent with respect to thermostability.

Seeing remaining activity after incubation at 65°C for 2 minutes, when phenylalanine at position 39 was substituted with isoleucine, leucine or valine, thermostability of modified GP was superior to that of natural potato-derived type L GP. Regarding substitution at position 39, substitution with leucine (remaining activity after incubation at 65°C for 2 minutes is 61.2%) was most excellent with respect to thermostability. When asparagine at

position 135 was substituted with alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, valine or tyrosine, thermostability of modified GP was superior to that of natural potato-derived type L GP. Regarding substitution at position 135, substitution with alanine (remaining activity after incubation at 65°C for 2 minutes is 79.0%), cysteine (remaining activity after incubation at 65°C for 2 minutes is 76.9%), glycine (remaining activity after incubation at 65°C for 2 minutes is 58.4%), methionine (remaining activity after incubation at 65°C for 2 minutes is 52.6%), serine (remaining activity after incubation at 65°C for 2 minutes is 86.5), threonine (remaining activity after incubation at 65°C for 2 minutes is 62.4%) or valine (remaining activity after incubation at 65°C for 2 minutes is 79.3%) was particularly excellent with respect to thermostability. When threonine at position 706 was substituted with cysteine, isoleucine, leucine, valine or tryptophan, thermostability of modified GP was superior to that of natural potato-derived type L GP. Regarding substitution at a position 706, substitution with leucine (remaining activity after incubation at 65°C for 2 minutes is 57.8%) or valine (remaining activity after incubation at 65°C for 2 minutes is 59.2%) was particularly excellent with respect to thermostability.

As a result, it was found that the thermostability of modified GP of the present invention was very improved as compared with natural potato type L GP.

(Example 3-2: Preparation of type H GP enzyme having improved thermostability)

(1) Large scale preparation of enzyme

Each of Escherichia coli expressing potato type H GP having improved thermostability and Escherichia coli expressing Arabidopsis thaliana type H GP having improved thermostability prepared in Examples 2-2B and 2-2C respectively was cultured in a TB medium (containing Terrific broth (GIBCO) 47 g/L, glycerol 4 ml/L and 50 μg/ml ampicillin) at 37°C for 5 hours, IPTG and pyridoxine hydrochloride were added to this culture solution to final concentrations of 0.1 mM IPTG and 1 mM pyridoxine hydrochloride, and this was further cultured at 22 °C for 24 hours. Then, bacterial cells were recovered by centrifuging the culture, and culture components were removed by washing with a 20 mM citrate buffer (pH 6.7). Bacterial cells after washing were suspended in a 20 mM citrate buffer (pH 6.7), bacterial cells were lysed with a sonicator, and centrifuged, and the supernatant was used as a bacterial cell extract. The resulting bacterial cell extract was purified using ion exchange chromatography and hydrophobic chromatography to recover a purified enzyme-containing active fraction exhibiting a single band by native PAGE (Native polyacrylamide gel electrophoresis).

(2) Comparison of thermostabilities of type H GP enzymes having improved thermostability

Thermostability at 60°C and 65°C of respective GPs having improved thermostability purified in (1) were compared. As a control, natural (not mutated) potato type H GP and Arabidopsis thaliana type H GP, purified by the same method, were used.

0.2U/ml of a purified enzyme solution (20 mM citrate

buffer (pH 6.7)) was incubated at 60°C for 10 minutes or 65°C for 2 minutes, and retained on ice. An enzyme solution retained on ice was 10-fold diluted with a 20 mM citrate buffer (pH 6.7), and enzyme activity was measured according to the activity measuring method described in Example 3-1 (2). Thermostability of an enzyme was judged by a ratio of enzyme activity at 37°C of the enzyme after incubation (i.e. remaining activity) when enzyme activity at 37°C of the enzyme before incubation at 58°C for 10 minutes, 60°C for 10 minutes or 65°C for 2 minutes is taken to be 100%. Results regarding potato type H GP having improved thermostability and natural potato type H GP are shown in the following Tables 8 and Fig. 11. Results regarding Arabidopsis thaliana type H GP having improved thermostability and natural Arabidopsis thaliana type H GP are shown in the following Tables 9 and Fig. 12.

Table 8

Temperature and time of incubation	Remaining activity (%) of natural potato type H GP	Remaining activity (%) of potato type H GP having improved thermostability (Y36L+N133S+T628I)
58°C 10 min	0	75.8
60°C 10 min	0	48.8
65°C 2 min	0	34.5

Table 9

Temperature and time of incubation	Remaining activity (%) of natural Arabidopsis thaliana type H GP	Remaining activity (%) of Arabidopsis thaliana type H GP having improved thermostability (Y40L+N136S+T631I)
58°C 10 min	1.5	48.8
60°C 10 min	0.5	29.3
65°C 2 min	0.5	29.2

From these results, potato type H GP having improved thermostability of the present invention had remaining an activity of 34.5% even after heating at 65°C for 2 minutes. On the other hand, natural potato type H GP had remaining activity of 0% after heating at 65°C for 2 minutes. From this, it was found that potato type H GP having improved thermostability of the present invention has high thermostability compared with natural potato type H GP.

In addition, Arabidopsis thaliana type H GP having improved thermostability of the present invention had remaining activity of 29.2% even after heating at 65°C for 2 minutes. On the other hand, natural Arabidopsis thaliana type H GP had remaining activity of 0.5% after heating at 65°C for 2 minutes. From this, it was found that Arabidopsis thaliana type H GP having improved thermostability of the present invention has high thermostability compared with natural Arabidopsis thaliana type H GP.

(Example 4: Synthesis of amylose of weight average molecular weight of 600 kDa or more, using α-glucan phosphorylase having improved thermostability)

Using α-glucan phosphorylase having improved thermostability according to the present invention, it was investigated whether amylose of a weight average molecular weight of 600 kDa or more can be synthesized. As an α-glucan phosphorylase having improved thermostability, any of the various GPs having improved thermostability (single mutant F39L, single mutant N135S, single mutant T706I, double mutant (F39L+N135S), double mutant (F39L+T706I), double mutant (N135S+T706I) and triple mutant (F39L+N135S+T706I)) prepared in above Example 3-1 (1) was used.

As a control, Bacillus stearothermophillus-derived α-glucan phosphorylase (also referred to as Bacillus stearothermophillus), and Thermus aquaticus-derived α-glucan phosphorylase (also referred to as Thermus aquaticus) were used.

An amylose synthesis reaction was performed at 50°C for 18 hours using a reaction solution having the composition described in the following Table 10.

Table 10

Reaction solution composition	
Maltotetraose(G4)	40 μM
Glucose-1-phosphate	250 mM
Acetate buffer(pH 5.5)	200 mM
α-glucan phosphorylase	4U/ml

A yield of amylose synthesized by this reaction was calculated by a calculation method described in 1.5 of the aforementioned "1. Measuring method and calculating method".

A weight average molecular weight of amylose synthesized by this reaction was measured by the following method. Amylose synthesized by this reaction was completely dissolved in 1N sodium hydroxide, and neutralized with a suitable amount of hydrochloric acid, about 30 to 300 μg of an aliquot of amylose was subjected to gel filtration chromatography using a differential refractometer and a multiangular light scatter detector together to obtain a weight average molecular weight.

More particularly, Shodex SB806M-HQ (manufactured by SHOWA DENKO K.K.) was used as a column and a multiangular light scatter detector (DAWN-DSP, manufactured by Wyatt Technology) was used as a detector, and a differential refractometer (Shodex RI-71, manufactured by SHOWA DENKO K.K.) were used by connecting them in that order. The column was retained at 40°C and, as an eluent, a 0.1M sodium nitrate solution was used at a flow rate of 1mL/min. The resulting signal was collected using a data analyzing software (trade name ASTRA, manufactured by Wyatt Technology), and this was analyzed using the same software, and a weight average molecular weight was thereby obtained. This method is also referred to as MALLS analyzing method.

The yields and a molecular weights of synthesized amylose obtained in this manner are shown in the following Table 11.

Table 11

Yield and molecular weight of synthesized amylose

α-glucan phosphorylase	Yield of amylose (%)	Molecular weight of amylose (kDa)
GP having improved thermostability (F39L)	51.2	668
GP having improved thermostability (N135S)	47.8	735
GP having improved thermostability (T706I)	45.3	675
GP having improved thermostability (F39L+N135S)	44.7	673
GP having improved thermostability (F39L+T706I)	47.5	706
GP having improved thermostability (N135S+T706I)	42.7	655
GP having improved thermostability (F39L+N135S+T706I)	52.3	645
Bacillus stearothrmophillus	17.3	20.0
Thermus aquaticus	27.8	44.3

As described above, it was found that the GP having improved thermostability according to the present invention can synthesize high-molecular amylose having a weight average molecular weight of about 600 kDa or more. In addition, it was found that the GP having improved thermostability according to the present invention has a yield of amylose of about 40% or more. Bacillus stearothermophillus GP and Thermus aquaticus GP used as Comparative Example are enzymes having thermostability, but cannot not synthesize high-molecular amylose.

(Example 5: Synthesis of amylose from sucrose using α-glucan phosphorylase having improved thermostability)

Using α-glucan phosphorylase having improved

thermostability according to the present invention, and using sucrose as a raw material, amylose was synthesized. As a α-glucan phosphorylase having improved thermostability, any of the various GPs having improved thermostability (single mutant F39L, single mutant N135S, single mutant T706I, double mutant (F39L+N135S), double mutant (F39L+T706I), double mutant (N135S+T706I) and triple mutant (F39L+N135S+T706I)) prepared in Example 3-1 (1) above was used.

An amylose synthesis reaction was performed at 50°C for 18 hours using a reaction solution of the composition described in the following Table 12.

Table 12

Reaction solution composition	
Sucrose	58.5mM
Maltotetraose (G4)	10 μM
Inorganic phosphoric acid (Pi)	10 mM
Sucrose phosphorylase	1 U/ml
α-glucan phosphorylase	1 U/ml

A yield (%) of amylose synthesized by this reaction was calculated by a calculation equation described in 1.7 of the aforementioned "1. Measuring method and calculating method".

A weight average molecular weight of amylose synthesized by this reaction was measured by the same method as that of Example 4 above. The yield sand weight average molecular weights of synthesized amylose obtained like this are shown in the following Table 13.

Table 13

Yield and weight average molecular weight of synthesized amylose

α-glucan phosphorylase	Yield of amylose (%)	Weight average molecular weight of amylose (kDa)
GP having improved thermostability (F39L)	50.8	672
GP having improved thermostability (N135S)	47.8	740
GP having improved thermostability (T706I)	44.6	675
GP having improved thermostability (F39L+N135S)	44.9	674
GP having improved thermostability (F39L+T706I)	47.5	707
GP having improved thermostability (N135S+T706I)	42.9	657
GP having improved thermostability (F39L+N135S+T706I)	52.3	649

As described above, it was found that the GP having improved thermostability according to the present invention can synthesize high-molecular weight amylose of about 600 kDa like natural GP, when amylose is synthesized using sucrose as a raw material. In addition, it was found that an amylose yield is high, such as about 40% or more, similar to natural GP.

(Example 6: Synthesis of glucan from glucose-1-phosphate, using GP having improved thermostability under high temperature condition (50°C, 55°C and 60°C))

Using a α-glucan phosphorylase having improved thermostability according to the present invention, and using

glucose-1-phosphate as a raw material, amylose was synthesized under high temperature conditions. GP having improved thermostability (triple mutant (F39L+N135S+T706I)) prepared in Example 3-1 (1) was used and, as a control, natural potato type L GP purified by the same method was used.

An amylose synthesizing reaction was performed by retaining a reaction solution containing G-1-P 6.1g/L, maltotetraose (G4) 0.3g/L, and GP 20U/L at 37°C, 50°C, 55°C or 60°C for 18 hours. An amount of synthesized amylose in the reaction product was measured over time. The amount of synthesized amylose (g/L) was calculated based on the following equation.

(Amount of synthesized amylose (g/L))

=(Glucose (mM) used in amylose synthesis)×180 (molecular weight of glucose)

=[(inorganic phosphoric acid (mM) produced by reaction)-(glucose (mM) after reaction)] × 180 (glucose molecular weight)

The amount of synthesized amylose after reaction for 18 hours is shown in the following Table 14 and Fig. 7.

Table 14

Amount of synthesized amylose (g/L)

Reaction temperature	Natural potato type L GP	GP having improved thermostability
37°C	2.8	3.1
50°C	3.2	3.3
55°C	2.5	2.7
60°C	0	1.5

When GP having improved thermostability was used, about 3g/L of amylose was synthesized at 37°C, 50°C and 55°C, and even about 1.5g/L of amylose was synthesized at 60°C. On the other hand, when natural potato type L GP was used, amylose was synthesized at 37°C, 50°C and 55°C, but amylose was not synthesized at all at 60°C. It is thought that, in natural potato type L GP, GP was inactivated at an initial stage of the reaction, at 60°C. On the other hand, it is thought that since GP having improved thermostability stably retained enzyme activity also at 60°C, an amylose synthesizing reaction was sufficiently performed. In addition, when GP having improved thermostability was used at each temperature of 37°C, 50°C, 55°C and 60°C, an amount of synthesized amylose was larger than that when natural potato type L GP was used. It is thought that the amount of synthesized amylose when GP having improved thermostability is used, is further increased as the reaction time is extended. As described above, it was found that GP having improved thermostability according to the present invention can synthesize a glucan at 60°C, at which temperature natural potato type L GP cannot react.

(Example 7 Synthesis of glucan from glucose-1-phosphate using GP having improved thermostability at 65°C and 70 °C)

Using GP having improved thermostability (triple mutant (F39L+N135S+T706I)) prepared in Example 3-1 (1) as in Example 6, a glucan was synthesized from glucose-1-phosphate under further high temperature conditions. As a control, natural potato type L GP was used.

By retaining a reaction solution containing G-1-P 15.2g/L, maltotetraose (G4) 2.7g/L, and GP 200U/L at 37°C, 65°C or 70 °C for 4 hours, an amylose synthesizing reaction was performed. The amount of synthesized amylose was calculated as in Example 6. After reaction time of 4 hours, when natural potato type L GP was used, amylose was not synthesized at all at 65°C and 70 °C, but when GP having improved thermostability was used, about 5.6g/L of amylose was synthesized from 15.2g of G-1-P at 65°C, and about 0.3g/L of amylose was synthesized from 15.2g of G-1-P at 70 °C. As a result, it was found that natural potato type L GP cannot synthesize amylose at 65°C to 70 °C, but GP having improved thermostability retains GP activity at a high temperature such as 70 °C, and has amylose synthesizing ability.

Based upon the results of Examples 6 and 7, it was found that the GP having improved thermostability according to the present invention has amylose synthesizing ability at high temperature conditions under which natural potato type L GP cannot react at all.

(Example 8: Confirmation of removal of contaminating protein by heat treatment)

It was confirmed the following method can be used to easily purify α-glucan phosphorylase having improved thermostability, using heat treatment.

Escherichia coli (TG-1) expressing GP having an improved thermostability (triple mutant (F39L+N135S+T706I)) gene prepared in Example 2-1A was cultured in a LB medium as in Example 2-1A. As a control, Escherichia coli (TG-1) expressing natural potato type L α-glucan phosphorylase was cultured in a LB medium as in Example 2-1A. Bacterial cells were recovered by centrifuging the culture solution, bacterial cells were suspended in a buffer, and this was sonicated to obtain a bacterial cell extract. This bacterial cell extract was heated in a water bath at 60°C for 0 to 60 minutes, and centrifuged to remove insoluble proteins to obtain the supernatant. GP activity and the protein content of this supernatant were measured, and the specific activity of a GP enzyme was obtained. GP activity was measured using the activity measuring method described in Example 3-1 (2), and the protein content was measured using the Bradford method (Bradford, M., Anal. Biochem., 72,248-254 (1976). The Bradford method is a colorimetric method in which a chromogenic substrate is bound to all proteins contained in a solution. In the present specification, measurement was performed using a protein assay kit (Nippon Bio-Rad Laboratories, Inc.) and bovine globulin used as a standard.

The specific activity of a GP enzyme was calculated by the following method.

Specific activity (U/ml)
=(α-glucan phosphorylase activity)/(mass mg of protein contained in supernatant)

Fig. 6 shows the change in specific activity with time of a GP enzyme having improved thermostability (referred

to as GP having improved thermostability (F39L+N135S+T706I in Fig. 6)) and natural potato type L GP enzyme.

As shown in Fig. 6, specific activity of GP having improved thermostability increased about 10-fold upon heating at 60°C. Contaminating proteins were almost completely thermally denatured and removed. To the contrary, specific activity of natural potato type L GP reduced with time. It is thought that this is because not only contaminating proteins but also the GP protein were denatured. In this manner, it was found that GP having improved thermostability can be simply purified by heat treatment.

(Example 9: Confirmation of removal of contaminating proteins by heat treatment)

As in Example 8, Escherichia coli (TG-1) expressing a gene of GP having improved thermostability (triple mutant (F39L+N135S+T706I)) was cultured, and a bacterial cell extract was prepared. Using this bacterial cell extract, it was confirmed that amylase activity and phosphatase activity can be reduced to a level which can be utilized in industrial production of amylose or G-1-P, by heat treatment at 60°C.

As in Example 8, a bacterial cell extract was heated in a water bath at 60°C for 30 minutes, and insoluble proteins were removed by centrifugation to obtain the supernatant. Phosphatase activity and amylase activity of this supernatant were measured.

Phosphatase activity was measured by retaining a reaction solution containing 100 μl of this supernatant and 100 μl

of 50 mM glucose-1-phosphate at 37°C for 60 minutes, and quantitating free inorganic phosphoric acid produced from glucose-1-phosphate in a reaction solution by a method described in (1. Measuring method and calculating method). An amount of an enzyme producing 1 μmol of inorganic phosphoric acid for one minute was defined as one unit (U). Amylase activity was obtained by retaining a reaction solution containing 25 μl of the supernatant and 25μl of 0.5% amylose (weight average molecular weight about 50 kDa) at 37°C for 60 minutes, adding 1ml of an iodine solution (0.1% potassium iodide, 0.01%), and measuring the reduction rate of iodine color development accompanied with conversion of amylose in a reaction solution into low-molecular amylose. Activity capable of reducing the absorbance at A660 by 10% for one minute was defined as 1U.

Amylase activity (U/min)
=(absorbance at A660nm before reaction-absorbance at A660nm after reaction) ÷ (absorbance at A660nm before reaction) × 100 ÷ 10 ÷ (time (min))

The following Table 15 shows a remaining ratio of phosphatase activity and amylase activity in a bacterial cell extract.

As shown in Table 15, when the activity of a bacterial cell extract before heating is taken to be 100%, phosphatase activity and amylase activity were such that phosphatase activity was about 3%, and amylase activity was about 0.3% after heating at 60°C, and these two contaminating proteins were almost inactivated.

Table 15

	Phosphatase activity (%)	Amylase activity (%)
Before heating	100	100
After heating at 60°C for 30 minutes	3.1	0.3

In this manner, α-glucan phosphorylase having improved thermostability according to the present invention is a plant GP enzyme which does not lose activity even after heat treatment at 60°C, and it was found that, by performing heat treatment at 60°C, it is possible to easily produce excellent GP which contains little amylase activity and phosphatase activity.

(Example 10: Stability of GP protein)

It has been reported that natural potato-derived type L GP proteins are easily degraded. These GPs, even when refrigerated after purification, are gradually degraded during storage. Generally, when an enzyme is degraded, a change in structure, a change in the nature of an enzyme, reduction in activity and the like occur. If the stability of a GP protein can be enhanced, then influence of the above factors will be reduced, and this is advantageous in terms of storage and use of an enzyme.

A natural potato-derived type L GP protein and seven kinds of GP proteins having improved thermostability (single mutant F39L, single mutant N135S, single mutant T706I, double mutant F39L+N135S, double mutant F39L+T706I, double mutant N135S+T706I, triple mutant F39L+N135S+T706I) prepared in Example 3-1 (1) were stored at 4°C, and the molecular weight of the GP protein was investigated over time for 5 months.

In addition, the molecular weight of a GP protein when stored at 37°C for 10 days was similarly investigated. Immediately after purification, and after storage at 4°C for 5 months, the molecular weight was investigated by polyacrylamide gel electrophoresis (Native-PAGE), and the results are shown in Fig. 13. The amount of a protein loaded onto a gel was equal for all GP proteins.

As a result, natural potato type L GP and seven kinds of GP having improved thermostability all showed a band at a position of a molecular weight of about 210 kDa (a monomer of GP has a molecular weight of about 104 kDa, and forms a dimer) immediately after purification . On the other hand, natural potato type L GP and a N135S mutant after storage at 4°C for 5 months had a molecular weight of about 140 kDa, which was smaller than that immediately after purification. This show that natural potato type L GP and a single mutant 135S were degraded during storage. Natural potato type L GP and a N135S mutant, also when stored at 37°C for 10 days, were degraded during storage. The other six kinds of GPs having improved thermostability (single mutant F39L, single mutant T706I, double mutant F39L+N135S, double mutant F39L+T706I, double mutant N135S+T706I, triple mutant F39L+N135S+T706I) after storage at 4°C for 5 months had a molecular weight of about 210 kDa, which is the same as that immediately after purification, and degradation of the proteins was not recognized. In addition, these six kinds of GPs having improved thermostability, also after storage at 37°C for 10 days, had no change in a molecular weight, and degradation of the GP protein was not recognized. This shows that these GPs having improved thermostability are excellent in degradation resistance, and have higher stability than natural potato type L GP, at between 4°C to

37°C. From this, it was found that substitution at a F39 position and substitution at a T706 position impart not only the effect of improving thermostability effect but also the effect of suppressing degradation to a GP protein.

(Example 11: Synthesis of glucose-1-phosphate)

(1) Synthesis of G-1-P using GP having improved thermostability at 65°C

Using a α-glucan phosphorylase having improved thermostability according to the present invention, and using a glucan and inorganic phosphoric acid as a raw material, G-1-P was synthesized at 65°C. GP having improved thermostability (triple mutant (F39L+N135S+T706I)) prepared in Example 3-1 (1) was used and, as a control, natural potato type L GP purified by the same method was used. A reaction solution containing 300 mM phosphate buffer (pH 7.0), 10g/L dextrin, and 1000U/L of any of the GP was retained at 37°C or 65°C for 18 hours, and a G-1-P synthesis reaction was performed. The amount of synthesized G-1-P was calculated by multiplying the G-1-P concentration (mM) obtained by the method of quantitating glucose-1-phosphate described in 1.3 of the "1. Measuring method and calculating method" above, by 260 which is a molecular weight of G-1-P. The amount of synthesized G-1-P after the reaction is shown in the following Table 16.

Table 16

Amount of synthesized glucose-1-phosphate (g/L)

Reaction temperature	Natural potato type L GP	GP having improved thermostability
37°C	3.5	4.2
65°C	0.0	3.7

When natural potato type L GP was used, G-1-P was not synthesized at 65°C. However, when GP having improved thermostability was used, it was possible to produce G-1-P even at 65°C.

(2) Synthesis of G-1-P using GP having improved thermostability, at 70 °C

As in Example 11 (1) above, using GP having improved thermostability according to the present invention and natural potato type L GP, and using a glucan and inorganic phosphoric acid as raw materials, G-1-P was synthesized at 70 °C. A reaction solution containing a 300 mM phosphate buffer (pH 7.0), 10g/L dextrin, and 10,000U/L of any of the GP was incubated at 70 °C for 4 hours, and G-1-P was synthesized. The amount of synthesized G-1-P was calculated as in the aforementioned Example.

When natural potato type L GP was used, G-1-P was not synthesized at all at 70 °C, but when GP having improved thermostability was used, about 1g of G-1-P was synthesized.

As described above, the present invention was exemplified using preferable embodiments of the present invention, but it should not be construed to limit the present invention to those embodiments. It is understood that the scope of the present invention should be construed only by claims. It is understood that those skilled in the art can practice an equivalent scope based on the description of the present invention and common technical knowledge, from the specific description of preferable embodiments of the present invention. It is understood that the content itself of patents, patent applications and references cited in the

present specification should be incorporated by reference, as if the content thereof are specifically described in the present specification.

INDUSTRIAL APPLICABILITY

According to the present invention, a plant-derived GP enzyme having excellent thermostability at a high temperatures (e.g. 60°C or higher) is obtained. α-glucan phosphorylase having improved thermostability of the present invention is useful in a glucan synthesizing reaction under high temperature conditions (e.g. 60°C or higher), under which a natural GP enzyme cannot react.

When a gene encoding α-glucan phosphorylase having improved thermostability according to the present invention (e.g. a gene encoding GP having improved thermostability obtained by improving thermostability of potato-derived GP) is highly expressed using a mesophilic bacterium such as Escherichia coli as a host, by heating a bacterial cell extract containing a enzyme having improved thermostability at 60°C, contaminating enzymes derived from a host bacterium can be simply removed. In particular, amylase activity and phosphatase activity, which are a great problem, particularly, during industrial utilization of a GP enzyme, can be considerably reduced by heat treatment. Therefore, the enzyme of the present invention is particularly useful in enzyme purification.

The method of the present invention is effective not only in potato derived GP and Arabidopsis thaliana derived GP, but also can be preferably applied to improving the thermostability of other group A GPs exhibiting high

homology to an amino acid sequence of potato derived GP or Arabidopsis thaliana derived GP. By using the method of the present invention, GP having improved thermostability derived from an organism species other than potato and Arabidopsis thaliana can be prepared.

According to the present invention, GP having improved thermostability, in which degradation of an enzyme protein is suppressed, and storage stability is improved, is provided.

(Explanation of Sequence Listing)

SEQ ID NO: 1: base sequence encoding potato type L α-glucan phosphorylase;

SEQ ID NO: 2: amino acid sequence of potato type L α-glucan phosphorylase;

SEQ ID NO: 3: base sequence encoding sweet potato type L α-glucan phosphorylase;

SEQ ID NO: 4: amino acid sequence of sweet potato type L α-glucan phosphorylase;

SEQ ID NO: 5: base sequence encoding potato second type L α-glucan phosphorylase;

SEQ ID NO: 6: amino acid sequence of potato second type L α-glucan phosphorylase;

SEQ ID NO: 7: base sequence encoding Fava bean type L α-glucan phosphorylase;

SEQ ID NO: 8: amino acid sequence of Fava bean type L α-glucan phosphorylase;

SEQ ID NO: 9: base sequence encoding Arabidopsis thaliana type L α-glucan phosphorylase;

SEQ ID NO: 10: amino acid sequence of Arabidopsis thaliana type L α-glucan phosphorylase;

SEQ ID NO: 11: base sequence encoding spinach type L α-glucan phosphorylase;

SEQ ID NO: 12: amino acid sequence of spinach type L α-glucan phosphorylase;

SEQ ID NO: 13: base sequence encoding corn type L α-glucan phosphorylase;

SEQ ID NO: 14: amino acid sequence of corn type L α-glucan phosphorylase;

SEQ ID NO:15: base sequence encoding rice type L α-glucan phosphorylase;

SEQ ID NO: 16: amino acid sequence of rice type L α-glucan phosphorylase;

SEQ ID NO: 17: base sequence encoding rice second type L α-glucan phosphorylase;

SEQ ID NO: 18: amino acid sequence of rice second type L α-glucan phosphorylase;

SEQ ID NO: 19: base sequence encoding wheat type H α-glucan phosphorylase;

SEQ ID NO: 20: amino acid sequence of wheat type H α-glucan phosphorylase;

SEQ ID NO: 21: base sequence encoding Citrus hybrid cultivar type H α-glucan phosphorylase;

SEQ ID NO: 22: amino acid sequence of Citrus hybrid cultivar type H α-glucan phosphorylase;

SEQ ID NO: 23: base sequence encoding rice type H α-glucan phosphorylase;

SEQ ID NO: 24: amino acid sequence of rice type H α-glucan phosphorylase;

SEQ ID NO: 25: base sequence encoding Fava bean type H α-glucan phosphorylase;

SEQ ID NO: 26: amino acid sequence of Fava bean type H α-glucan phosphorylase;

SEQ ID NO: 27: base sequence encoding Arabidopsis

thaliana type H α-glucan phosphorylase;

SEQ ID NO: 28: amino acid sequence of Arabidopsis thaliana type H α-glucan phosphorylase;

SEQ ID NO: 29: base sequence encoding potato type H α-glucan phosphorylase;

SEQ ID NO: 30: amino acid sequence of potato type H α-glucan phosphorylase;

SEQ ID NO: 31: a partial sequence of a base sequence encoding sweet potato type H α-glucan phosphorylase;

SEQ ID NO: 32: amino acid sequence of sweet potato type H α-glucan phosphorylase;

SEQ ID NO: 33: base sequence encoding potato type L α-glucan phosphorylase having improved thermostability;

SEQ ID NO: 34: amino acid sequence of potato type L α-glucan phosphorylase having improved thermostability;

SEQ ID NO: 35: amino acid sequence of Escherichia coli maltodextrin phosphorylase;

SEQ ID NOS: 36 and 37: base sequence around a linking site with a plasmid pMW118 shown in Fig. 2;

SEQ ID NO: 38: base sequence of PCR primer 1;

SEQ ID NO: 39: base sequence of PCR primer 2;

SEQ ID NO: 40: base sequence of PCR primer 3;

SEQ ID NO: 41: base sequence of PCR primer 4;

SEQ ID NO: 42: base sequence of PCR primer 5;

SEQ ID NO: 43: base sequence of PCR primer 6;

SEQ ID NO: 44: amino acid sequence of motif sequence 1L;

SEQ ID NO: 45: amino acid sequence of motif sequence 1H;

SEQ ID NO: 46: amino acid sequence of motif sequence 2;

SEQ ID NO: 47: amino acid sequence of motif sequence 3L;

SEQ ID NO: 48: amino acid sequence of motif sequence 3H.